University of Alberta

In vivo and *in vitro* evaluation of *Beauveria bassiana* pathogenicity for western flower thrips, *Frankliniella occidentalis*

by

Sunil Rajput © ©

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Dedication

This thesis is dedicated with Love and Respect

to my

Father, Mother and my two Brothers – Sunjay and Vinay.

Abstract

Western flower thrips, *Frankliniella occidentalis*, are major pests in greenhouses. The fungus *Beauveria bassiana* is a safer alternative to use of chemical insecticides against this insect; and evaluating isolates for high virulence has usually involved *in vivo* studies. My approach examines *in vitro* properties of *B. bassiana* in relation to *F. occidentalis in vivo* virulence. I developed *in vitro* tools based on colony growth and metabolite analyses to identify isolates for biocontrol potential. Six characteristics were developed and assessed against LT_{50} data. My results indicated that 3 combined characteristics (harvest quality, mycelial fragments and degree of submerged mycelia) can be used to predict virulence. Oosporein production was also examined by photo-exposure. Results indicated that all 4 isolates tested, exhibiting low to high virulence, produced oosporein thereby disassociating it from whole animal virulence. Instead, oosporein may function as a photo-protectant. Beauvericin production was also evaluated by HPLC and was produced by all 5 isolates tested. Beauvericin production and whole animal virulence appear to be uncoupled although my results do suggest a weak correlation. The toxicity of beauvericin was evaluated on a whitefly embryonic cell line and was found to be sensitive in a dose- and time-dependent fashion. I demonstrated the CC_{50} after 48 h incubation to be comparable to that of other researchers using different cell lines. These analyses will help reduce processing time and labour intensity required for *in vivo* studies when screening isolates. This approach contributes to a greater understanding of pathology and supports microbial control registration for commercial use.

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List of abbreviations

For all tables: values presented in scientific notation as nE+d are equivalent to the value n x 10^d.

°C – degrees Celsius.

AAFC – Agriculture and Agri-Food Canada.

ANOVA – analysis of variance.

ARC – Alberta Research Council (Vegreville, Alberta).

BEA – beauvericin.

charact. – characteristics.

chromato. – chromatogram.

cm – centimetre.

DNA – deoxyribonucleic acid.

evalu. – evaluation.

GLM – generalized linear model.

gm – gram.

h – hour.

HPLC – high-performance liquid chromatography.

IPM – integrated pest management.

L:D – ratio of light to dark time periods.

liq. – liquid.

m – metre.

MIC – minimum inhibitory concentration.

min. – minute.

mm – millimetre.

N/D – non-discernable response.

nm – nanometre.

PI – propidium iodide.

PMRA – pest management regulatory agency.

RBSS – Rinaldini's balanced salt solution.

rRNA – ribosomal ribonucleic acid.

SDAY – Sabouraud dextrose agar with yeast extract.

SEM – scanning electron micrograph.

std – standard.

TLC – thin layer chromatography.

UV – ultraviolet.

viab. – viabilities.

WFT – western flower thrips.

YE – yeast extract

Chapter 1. Literature review

1.1. Introduction

A Green Revolution, begun in the 1960s to save humans from starvation, used selected cultivars of crop plants grown in conjunction with application of chemical fertilizers, pesticides and controlled irrigation to produce greater yields than those resulting from use of conventional cultivars and methods (Rosset *et al.*, 2000). This Revolution showed potential, but as time advanced, employing such methods became detrimental. For example, replacing conventional cultivars and traditional methods with modern ones caused yields to decline in less developed regions such as with rice yields in central Luzon, Philippines since the 1970s (Rosset *et al.*, 2000). With a constant increase in production costs, many smaller producers were forced to retire and those able to accommodate increasing costs accumulated more production area to maximize profits (Rosset *et al.*, 2000). Large-scale application of chemical pesticides resulted in insect populations developing resistance to them over time (Lacey and Goettel, 1995). Eliminating natural enemies through intensive chemical application caused pest resurgence, secondary pest outbreaks and the development of non-pest insects to pest population status thus upsetting nature's balance (Lacey and Goettel, 1995). Use of chemical agents has other important consequences that affect humans and the environment such as persistent residues and contamination of groundwater (Lacey and Goettel, 1995; Inglis *et al.*, 2001). Due to limited alternatives, many of these methods are still employed in intensive cropping systems in the agricultural and horticultural industries worldwide.

1.2. Greenhouse market

1.2.1. *Operational scale*

In 2001, the total area under glass or plastic in Canada was 18,352,644 m^2 (Fry, 2003; Sparkes and Irving, 2004). From 1991 to 2001, this area more than doubled, expanding by 9.5 million m^2 (Sparkes and Irving, 2004). In 2001, the Canadian floriculture industry generated $1.3 billion from both domestic and export sales (Sparkes and Irving, 2004). More than 40% of Canadian greenhouse flowers and other crops are marketed to domestic wholesalers or sold directly to the public (Sparkes and Irving, 2004). The export market for flowers, though still small (11% of total sales in 2001), has grown strongly in recent years (Sparkes and Irving, 2004). Farm cash receipts from greenhouse vegetables in 1997 were $269 million (Fry, 2003). Vegetable sales in British Columbia in 2002 were $200 million (Fry, 2003). In 2004, Alberta greenhouse produce value was estimated at $140 million with an investment of over $260 million (Mirza *et al*., 2006). These values indicate that the greenhouse market contributes significantly to Canada's economy. Figures 1.1 – 1.3 are examples of the operational scale.

The United States greenhouse production is larger due to a larger consumer market and to outdoor production with estimated sales of nearly $14 billion for floriculture products in 1997 (Fry, 2003). Forty percent of all floriculture products sold in the U.S. are grown there; thus a large and very vibrant horticulture industry exists in North America (Fry, 2003).

A 1993 survey of Alberta greenhouse and nursery growers suggested that of the growers that responded, 51% would be expanding their business within the next 1-5 years

(Fry *et al.*, 1999; Fry, 2003). In 2001, the total greenhouse product sales in Canada reached $1.86 billion, a 93% increase from 1996 (Sparkes and Irving, 2004). This suggests that the greenhouse industry is capable of sustaining itself in the marketplace and that most growers intend to further advance this sector. However, Fry *et al.* (1999) explained that in 1993, one of the growers' recommendations was the need for research to focus on biological control (Maan, 2001). Greenhouse and nursery growers intend to expand the greenhouse industry but as Fry *et al.* (1999) stated, growers in Alberta have expressed a pressing need for additional pest management tools.

1.3. Regulatory environment

1.3.1. Pest Management Regulatory Agency in Canada

Health Canada's Pest Management Regulatory Agency (PMRA) regulates all products used, sold or imported into Canada that are designed to manage, destroy, attract or repel pests (PMRA, 2001). Established in 1995, the PMRA seeks to minimize risks associated with pesticides, and thus to protect human health, safety and the environment (PMRA, 2001). For a pesticide to be used in Canada, it must undergo rigorous testing to determine potential risks to human health and the environment (PMRA, 2001).

As this regulatory system is tailored to the static nature of chemical insecticides, extended evaluation times are required to evaluate dynamic microbials due to the data requirements. With continuous pressure from the scientific community and greenhouse growers for microbial use, PMRA has joined with Agriculture and Agri-Food Canada (AAFC) to initiate a program for minor use registration of microbial-based insecticides. This program allows greenhouse growers to access a wider range of microbials more quickly for (initially) small-scale use, and promotes research efforts directed towards a safer and sustainable environment.

1.4. *Frankliniella occidentalis* (western flower thrips)

1.4.1. General biology and taxonomy

The English common name, thrips, is the Greek word for "woodworm," derived from observations by early naturalists on species found on dead branches (Mound, 2005). Thrips are small, opportunistic, vagile, ubiquitous insects mostly a few millimetres or less in length and generally yellow, brown or black (Morse and Hoddle, 2006). They can range from nondescript to species with exaggerated secondary sexual characteristics (Morse and Hoddle, 2006). Thrips are classified in the Order Thysanoptera, a name referring to the fringed wings of adults (Lewis, 1997; Mound, 2005). *Frankliniella occidentalis* (Pergande), the western flower thrips, belongs to the suborder Terebrantia, Family Thripidae and subfamily Thripinae (Lewis, 1997) (Figure 1.4).

1.4.2. Life history

Many species of thrips are preadapted to an invasive lifestyle (Morse and Hoddle, 2006). Western flower thrips were indigenous to western North America until 1980 (Mound, 1997), but have since disseminated worldwide, becoming a major pest (Kumm, 2002; Kirk and Terry, 2003).

Using an external, well-developed, serrated ovipositor to pierce the plant epidermis, adult female western flower thrips lay their eggs singly directly into plant tissue (Terry, 1997) (Figure 1.5). Terebrantians will oviposit into most plant tissues, although some species prefer oviposition sites such as the sides of the main veins on the underside of American basswood leaves (Terry, 1997). As oviposition involves direct damage to the plant, a cosmetic blemish, an economic loss to producers of many crops, results (Terry, 1997). Under optimum environmental and nutritional conditions, a female *F. occidentalis* can produce 30-300 eggs during her lifetime (Lewis, 1997; McDonough *et al*., 2002). *Frankliniella occidentalis* eggs are oval to kidney shaped and the chorion is smooth to reticulate and pale-white to brown (Terry, 1997) (Figure 1.4). Based on its development, *F. occidentalis* is classified as intermediate between holometabolous and hemimetabolous (Lewis, 1973; Heming, 1991). Postembryonic development of *F. occidentalis* consists of two active feeding larval instars and two relatively inactive, non-feeding, quiescent, pupal or metamorphic instars called a propupa and pupa (Kumm, 2002) (Figure 1.4). Adult females are larger than males and have more distinct colour patterns (Figure 1.4). Feeding damage is caused by both larval and adult thrips. The entire lifecycle ranges from 10 to 30 days, depending on temperature, day length and available food (Kumm, 2002). Adults can live for an additional 10 to 30 days (Lewis, 1997). In optimal environments for breeding, such as in warm regions or a greenhouse, 12 to 15 generations per year are possible. In cooler regions only 1 to 2 generations are possible with larvae overwintering in the soil or as adults in plant litter or under tree bark (Lewis, 1973, 1997; Kirk, 1997a). *Frankliniella occidentalis* occurs mainly in flowers, where larvae and adults obtain pollen contents, nectar and other plant fluids using piercing-sucking mouthparts (Higgins, 1992).

Thrips have unique, asymmetric mouthparts consisting of the left mandible, a pair of interlocking maxillary stylets which open apically into a feeding tube and an extrudable labral pad fringed by numerous papillae (Chisholm and Lewis, 1984; Hunter and Ullman, 1989). The stylets are enclosed in a mouthcone on the underside of the head formed by the labrum, maxillary stipites and labium (Kirk, 1997b). To feed, the mandible is protracted to pierce a hole in the epidermis and the maxillary stylets interlock to form a feeding tube by which cell contents are removed by action of a cibarial pump (Chisholm and Lewis, 1984; Hunter and Ullman, 1989; Kirk, 1997b).

Plants quickly dehydrate through regions of epidermis damaged by *F. occidentalis* feeding. If plants survive attack, damaged regions become distorted with subsequent growth, the most common result being curled leaves and rusted or speckled fruits (Lewis, 1973) (Figures 1.6 – 1.7). On mature leaves, light infestations result in silvering and scarring in patches on the laminae (Figure 1.7) or streaks along the veins; heavy infestations on leaves result in drying, withering and then shedding (Lewis, 1973). Fruitlets attacked early in development are fed on around the calyx or under the sepals, resulting in a ring-shaped scar about the base of the fruit (Lewis, 1973). Fruit becomes deformed as epidermal scars remain their initial size while, the remainder continues to grow (Figure 1.6); older fruits develop cracked scars (Lewis, 1973).

The western flower thrips feeding mode allows several disease-causing viruses to be transmitted. The commonly known tomato spotted wilt and impatiens necrotic spot tospoviruses, both in the Family Bunyaviridae, Genus *Tospovirus*, are transmitted by the piercing-sucking mode of feeding (Ullman *et al*., 1997). Recently, *F. occidentalis* has been implicated also in the transmission of pollen-borne viruses such as pelargonium flower break carmovirus, prune dwarf ilarvirus and prunus necrotic ringspot ilarvirus, through oral or bodily contact with virus-contaminated pollen and subsequent transmission through mechanical wounding of the plant during feeding (Greber *et al*., 1991a,b, 1992; Krczal *et al*., 1995; Ullman *et al*., 1997).

1.4.3. Economic significance: pest status

Western flower thrips are major pests in the greenhouse ornamental and vegetable industries (Lewis, 1997; Murphy *et al.*, 1998; Kumm, 2002). Characteristics of their biology that predispose them to become pests are: their ability to cause direct feeding and oviposition damage or, to spread virus diseases to food, fibre and ornamental crops; their tendency to spread and colonize widely, mainly by flight but also aboard planes and ships; and their ability to multiply rapidly in favourable environments (Lewis, 1997). In the Netherlands, western flower thrips has become the key pest of ornamental and vegetable crops in greenhouses (Kumm, 2002). It exhibits a broad host plant range (59 genera and 219 species of vegetable and ornamental crops) leading plant protection authorities to quarantine this pest species in the European Union (Kumm, 2002). *Frankliniella occidentalis* can cause up to 90% loss of summer-replanted cucumbers in United Kingdom greenhouses worth up to £50,000 ($US75,000) ha^{-1} each year (Lewis, 1997) and damage to sweet peppers in Florida caused by *F. occidentalis* and *Thrips palmi* in 1993 exceeded $US10 million (Nuessly and Nagata, 1995; Lewis, 1997).

Considering the host range, degree of direct feeding/oviposition damage and indirect damage through spread of viral diseases, the hundred-plus pest species in the family Thripidae (Lewis, 1997) together with the ability of thrips to rapidly infest leads to serious yield depletion. Revenue losses are proportional to damage inflicted so growers have a low tolerance for thrips infestations.

Though chemical insecticides are currently employed to manage thrips (Murphy *et al.*, 1998), use of such agents results in negative consequences such as insecticide resistance (Immaraju *et al.*, 1992; Brødsgaard, 1994; Kogel *et al.*, 1997) and concerns over harmful effects on the environment and humans (Inglis *et al.*, 2001). Safer alternatives to thrips management must be developed to limit chemical use and to reduce the risk of environmental and human contamination (Liu *et al.*, 2002).

1.5. Alternatives to chemical insecticides

1.5.1. Integrated Pest Management (IPM)

IPM originated in the late 1800s when ecology was identified as the foundation for scientific plant protection (Kogan, 1998). Use of IPM arose during the second half of the twentieth century (Kogan, 1998). Before this, knowledge of pest biology and of cultural and mechanical practices were used to protect crops from pests in the absence of pesticides (Kogan, 1998). Cultural and mechanical practices were IPM but not recognized as such at the time. When use of organosynthetic insecticides emerged in the early 1940s, the focus shifted from knowledge of pest biology and use of noninsecticidal methods to reliance on chemical control methods leading to the "dark ages" of the late 1940s to the mid 1960s (Kogan, 1998). By the mid 1940s, the risks associated with chemical insecticide overuse began to be recognized (Strickland, 1945; Kogan, 1998). The idea of "integrated control" was based on the complementary use of biological and chemical control methods (Michelbacher and Bacon, 1952; Kogan, 1998). The concept of "pest management" as distilled by Geier (1966) was: "a term used as a convenient label coined to convey the idea of intelligent manipulation of nature for humans' lasting benefit" (Kogan, 1998). In 1972, "integrated pest management" and its acronym IPM was accepted by the scientific community and included in the English literature (Kogan, 1998).

A recent survey recorded 64 definitions of integrated control, pest management or integrated pest management (Kogan, 1998). According to the Science Citation Index, Stern *et al.*'s (1959) definition for integrated control is most often cited (Kogan, 1998). A broader definition adopted by the Food and Agriculture Organization (FAO) states:

> "Integrated Pest Control is a pest management system that, in the context of the associated environment and the population dynamics of the pest species, utilizes all suitable techniques and methods in as compatible a manner as possible and maintains the pest population at levels below those causing economic injury" (Kogan, 1998).

This definition is cited frequently, but as with most definitions, an entomological bias in IPM is perpetuated because of the emphasis on pest populations and economic injury levels (EIL). This entomological bias reduces the application of the definition to plant pathogens and the idea of the action threshold is incompatible with pathogen epidemiology or with many weed management systems (Kogan, 1998). Kogan (1998) suggested a more balanced definition based on an analysis of definitions coined over the past 35 years stating:

> "Integrated Pest Management is a decision support system for the selection and use of pest control tactics, singly or harmoniously coordinated into a management strategy, based on cost/benefit analyses that take into account the interests of and impacts on producers, society, and the environment."

1.5.2. Microbials

Improved performance and cost competitiveness of microbial insecticides in conjunction with increasing, arthropod resistance to chemical insecticides, environmental concerns and lack of new chemical agents have greatly promoted the importance of microbials in pest management (Starnes *et al.*, 1993). Use of microbial control agents is growing at a rate of 10-25% per year compared with that of chemical insecticides of 1-2% per year (Starnes *et al.*, 1993). Due to their selectivity and minimal environmental impact, such agents are ideal components of integrated pest management programs (Lacey and Goettel, 1995).

There are five types of microbial insecticides: bacteria, protists, viruses, nematodes and fungi (Lacey and Goettel, 1995). Entomopathogenic fungi are more effective than other microbials as they do not need to be ingested and offer the only microbial-based control option against plant sucking insects (Lacey and Goettel, 1995). Commonly known examples of entomopathogenic fungi include *Metarhizium anisopliae* (Metschnikoff) Sorokin, *Verticillium lecanii* (Zimmermann) Viegas, *Beauveria brongniartii* (Saccardo) Petch, *Beauveria bassiana* (Balsamo) Vuillemin and *Paecilomyces fumosoroseus* (Wize) Brown and Smith.

1.6. *Beauveria bassiana*

1.6.1. Historical perspective

Beauveria bassiana is named after Agostino Bassi (1773-1856). In 1807, Bassi investigated the muscardine disease of silkworms, *Bombyx mori* (Linnaeus) resulting in several unsuccessful attempts to control it during which time he suffered from chronic illness. However he eventually discovered an "extraneous germ", a parasitic fungus, and published his results in 1835 (Ainsworth, 1956). Bassi (1835) described *Beauveria* as the causal agent of *mal del segno* or the mark disease, also known as *calcinaccio* or *cannellino* in Italy and as white *muscardino* in France, which caused economically devastating epizootics of domestic larval silkworms in southern Europe during the 18th and 19th centuries (Rehner and Buckley, 2005). Bassi was first to show that microbes act as contagious pathogens of animals, providing an important antecedent to the germ theory of disease (Ainsworth, 1956; Rehner and Buckley, 2005).

1.6.2. Taxonomy

The first taxonomic recognition of the *muscardino* fungus was proposed by Balsamo-Crivelli (1835a,b) who acknowledged Bassi's discoveries by naming this pathogen *Botrytis bassiana*. The genus *Beauveria* was not formally described until the early 20th century by Vuillemin (1912), who designated *Botrytis bassiana* Balsamo-Crivelli as the type species (Rehner and Buckley, 2005).

Until the recent discovery of its sexual or teleomorphic stage, *B. bassiana* had been placed in the phylum Deuteromycota (Fungi Imperfecti), class Hyphomycetes, for fungi having no known sexual stage, although they have other mechanisms for exchanging genetic material (Ainsworth *et al.*, 1983). Recently, in the People's Republic of China, a *Cordyceps* sexual stage was discovered aptly named *Cordyceps bassiana* (Li *et al.*, 2001). As mycologists emphasize the teleomorphic stage for taxonomic purposes, *B. bassiana* has been re-classified in the phylum Ascomycota, class Pyrenomycetes, order Hypocreales, family Clavicipitaceae (Rehner and Buckley, 2005). Although the sexual stage has been ascribed, *B. bassiana* usually occurs in its asexual or anamorphic stage and reproduces by mitotically producing asexual propagules called conidia (Figure 1.8).

1.6.3. Host range and distribution

Beauveria bassiana exhibits a broad host range including western flower thrips, *Frankliniella occidentalis* (Fry *et al.*, 1999; Fry, 2003), sweetpotato whitefly, *Bemisia tabaci* (Gennadius) (Zaki, 1998), silverleaf whitefly, *Bemisia argentifolii* (Bellows and Perring) (Wraight *et al.*, 1998), eastern tent caterpillar, *Malacosoma americanum* (Fabricius) (Leathers and Gupta, 1993), tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Steinkraus and Tugwell, 1997), diamondback moth, *Plutella xylostella* (Linnaeus) (Shelton *et al.*, 1998) and the grasshopper, *Melanoplus sanguinipes* (Fabricius) (Jaronski and Goettel, 1997). *Beauveria bassiana* is naturally present in soil and widely distributed geographically (St. Leger *et al.*, 1992). It has been isolated from soil samples in Canada (Alberta (Inglis *et al.*, 1992; Fry *et al.*, 1999; Fry, 2003; Litwinowich, 2005) and Ontario (Bidochka *et al.*, 1998)), the United States (Maine, Idaho, Washington, Vermont, North and South Carolina and Wisconsin (Brownbridge *et al.*, 1993; Wraight *et al.*, 1998)), subantarctic Macquarie Island (Roddam and Rath, 1997), Denmark (Steenberg *et al.*, 1995) and Finland (Vänninen *et al.*, 2000).

1.6.3.1. Variability

Beauveria bassiana is a cosmopolitan entomopathogen and although mitosporic; variations are known within and among isolates (St. Leger *et al.*, 1992; Maurer *et al.*, 1997; Castrillo and Brooks, 1998; Castrillo *et al.*, 1999). Genetic variation among 107 strains of *B. bassiana* originating from the darkling beetle, *Alphitobius diaperinus*

(Panzer) was surveyed using Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) and resulted in variation being revealed not only within and among strains from different regions but also among strains collected from a given insect host (Castrillo and Brooks, 1998; Castrillo *et al.*, 1999). Castrillo *et al.* (1999) suggested that its degree of intra-strain variability is greater than its inter-strain variability from different geographical locations.

Genetic recombination within *B. bassiana* is primarily due to action of the parasexual cycle whereby the presence of two different types of nuclei in the mycelium (heterokaryosis) may result in fusion to produce an unstable diploid nucleus (Pontecorvo, 1956; Alexopoulos *et al.*, 1996). This condition can occur by mutation or by fusion of mycelia with different types of nuclei (Pontecorvo, 1956; Alexopoulos *et al.*, 1996). Diploid nuclei can spontaneously generate diploid recombinants by mitotic crossing over and/or haploid recombination by non-disjunction, both events occurring at low frequencies (Pontecorvo, 1956; Paccola-Meirelles and Azevedo, 1991; Alexopoulos *et al.*, 1996). Genetic variability among isolates of *B. bassiana*, translates to varying degrees of virulence towards target insects. Inherent variability in the production of cuticle-degrading enzymes among isolates of *B. bassiana* also has a strong influence on isolate virulence (Gupta *et al.*, 1992, 1994). Even though *B. bassiana* exhibits a broad host range, individual isolates have various degrees of host specificity (Goettel, 1992). As a result, individual isolates of *B. bassiana* are routinely screened against target insects for efficacy and virulence.

1.6.4. Pathogenesis

Usually, the infective unit for entomogenous fungi is the conidium, an asexual propagule (Figure 1.8). *Beauveria bassiana* initiates an infection topically when exposed to a susceptible insect host. In some cases, entry can also be made via the alimentary tract (Broome *et al*., 1976) or respiratory system (Clark *et al*., 1968; Hedlund and Pass, 1968; Pekrul and Grula, 1979). However, emphasis will be placed on the external cuticular route due to it being most common. Susceptible insects come into contact with conidia that attach to host cuticle by hydrophobic interactions through action of presumed hydrophobic proteins (hydrophobins) in the cell wall of the conidia (Hajek and St. Leger, 1994; Kershaw and Talbot, 1998). If the amount of inoculum is large, conidia will adhere to all regions of the cuticle and clumping may occur (Pekrul and Grula, 1979). Once attached, an exogenous, utilizable, carbon-energy source is necessary for germination to occur. Though conidia have sufficient endogenous nitrogen reserves for short-term hyphal growth, additional nitrogen must be present to sustain further growth and extension of hyphae and to prevent autolysis (Smith and Grula, 1981). Germination is facilitated by high relative humidity but this is not essential (Ferron, 1977). Conidia germinate on all regions of the cuticle so that several infections can occur simultaneously. Hyphae (germ tubes) from germinating conidia exhibit positive chemotaxis toward the cuticle and penetrate it as early as 16-18 h post inoculation (Pekrul and Grula, 1979). During penetration, fungal enzymes are locally secreted at the growing tip of the germ tube resulting in a circular hole at point of entry without formation of any specialized appressorial-like structures, although some germ tubes exhibit thickenings (Pekrul and Grula, 1979). Proteases, lipases and a chitinase are secreted in sequential order reflecting the structure of the host integument and thus increasing efficiency of penetration (Samsinakova *et al*., 1971; Gupta *et al*., 1992, 1994).

Once breached (as early as 48 h post inoculation), the fungus proliferates (Pekrul and Grula, 1979). Hyphae are septate and produce yeast-like hyphal bodies for efficient nutrient uptake and secondary metabolite secretion many of which promote host tissue degradation (Roberts and Humber 1981; Goettel 1992; Fuguet and Vey, 2004).

Beauveria bassiana is known to produce at least five, low-molecular-weight, mainly cyclic, secondary metabolites (mycotoxins) exhibiting antibiotic or insecticidal properties (Strasser *et al.*, 2000; Vey *et al.*, 2001; Quesada-Moraga and Vey, 2004): beauvericin (Hamill *et al.*, 1969), bassinolide (Suzuki *et al.*, 1977), beauverilide (Isogai *et al.*, 1978), and bassianin and tenellin (two non-peptide toxins) (Wat *et al.*, 1977). Recently, a high-molecular-weight toxic protein metabolite, bassiacridin, has been purified and characterized to be toxic to locusts, *Locusta migratoria* (Linnaeus) (Quesada-Moraga and Vey, 2004). *Beauveria bassiana* is also known to produce pigments *in vitro* and/or *in vivo*. Emphasis is commonly placed on the reddish pigment, oosporein (Eyal *et al.*, 1994), as it is produced both *in vivo* and *in vitro* and has supposed antibacterial properties (Vining *et al.*, 1962; Brewer *et al.*, 1984; Taniguchi *et al.*, 1984; Wainwright *et al.*, 1986).

Beauveria bassiana post-infection activities include a rapid decrease in protein and carbohydrate concentration, physical invasion of the alimentary tract, and toxin production causing organ failure and cessation of feeding (Cheung and Grula, 1982). Starvation is not the ultimate cause of death. Instead, the physiological stress caused by infection (starvation, depletion of critical haemolymph constituents and toxemia), immune system failure (allows invading fungus to rapidly colonize the host) and toxin production resulting in terminal mycosis-all cause death (Cheung and Grula, 1982; Fuguet and Vey, 2004). Usually, the host insect dies within a few days. Time to death depends on dose and strain-specific virulence (Inglis *et al.*, 1997). Secondary bacterial infection is also possible during cuticle invasion, especially if this occurs during larval moulting (Poprawski *et al.*, 1997).

As host nutrients are exhausted, *B. bassiana* begins to shift from vegetative to reproductive growth. At this point, if relative humidity is suitable (≥92%), *B. bassiana* will re-emerge from host cuticle first at intersegmental regions, followed by elsewhere on the body. The fungus will then produce conidiophores bearing reproductive conidia that are later disseminated by abiotic and/or biotic factors to repeat the lifecycle (Ferron 1977; Pekrul and Grula, 1979) (Figure 1.9). If relative humidity is unsuitable at the time when nutrients are depleting, *B. bassiana* will remain dormant (i.e. mummification) until conditions improve to produce its reproductive structures (Roberts and Humber 1981).

1.6.4.1. Beauvericin

Beauvericin is the most studied of the mycotoxins produced by *B. bassiana*. It, initially, was reported to be synthesized by entomopathogenic fungi such as *B. bassiana* (Hamill *et al.*, 1969) and *P. fumosoroseus* (Bernardini *et al.*, 1975; Logrieco *et al.*, 2002a). Subsequently, it has been detected in numerous entomopathogenic and phytopathogenic isolates of *Fusarium* species (Gupta *et al.*, 1991; Logrieco *et al.*, 1993a; Logrieco *et al.*, 1993b; Moretti *et al.*, 1995; Logrieco *et al.*, 1998; Logrieco *et al.*, 2002b). Beauvericin ($C_{45}H_{57}N_3O_9$) is a lipophilic cyclic hexadepsipeptide consisting of repeating D-α-hydrossyisovaleryl-L-*N*-methyl-phenylalanine units and has a molecular weight of 784 (Hamill *et al.*, 1969; Thakur and Smith, 1997). It belongs to the family of enniatins, which exert antibiotic activities on Gram-positive bacteria and mycobacteria (Logrieco *et* *al*., 2002a). Antimicrobial properties associated with beauvericin are due to its catalyzing the translocation of cations across the lipid bilayer in the direction of the electrochemical gradient (Thaker and Smith, 1997). Beauvericin has ionophoric properties by forming complexes with monovalent and divalent cations that activates their transport into mammalian mitochondria and through biological and artificial membranes (Logrieco *et al*., 2002a). Beauvericin also induces programmed cell death (apoptosis) via calcium-activated endonucleases causing cytolysis accompanied by internucleosomal DNA fragmentation into multiples of 200 base pairs (Ojcius *et al*., 1991). Beauvericin has been shown to exhibit varying degrees of toxicity to invertebrates such as the crustacean, *Artemia salina* (Linnaeus) and the mosquito, *Aedes aegypti* (Linnaeus) (Hamill *et al*., 1969; Grove and Pople, 1980) and to invertebrate cell cultures such as *Spodoptera frugiperda* SF-9, a cell line derived from pupal ovaries (Calò *et al*., 2003; Fornelli *et al*., 2004). Beauvericin is also cytotoxic to several mammalian cell lines such as myeloid cells and cervical carcinoma HeLa cells, at concentrations in the lower micromolar range (Logrieco *et al*., 1996; Logrieco *et al*., 2002a; Calò *et al*., 2004). Due to its significance as a natural contaminant of *Fusarium*-infected cereal crops and as an active metabolite of many entomopathogenic fungi, beauvericin levels are of concern to pesticide regulatory agencies such as the Pest Management Regulatory Agency (PMRA), of Health Canada, when considering a biological agent such as *B. bassiana* for insect pest control.

1.6.4.2. Oosporein

Many soil fungi and entomogenous fungi belonging to the genus *Beauveria* produce a red-coloured pigment known as oosporein (Eyal *et al*., 1994; Strasser *et al*., 2000; Vey *et al*., 2001). During the infection process, when the host insect dies and prior to fungal re-emergence, the cadaver may turn red due to production of oosporein, a dibenzoquinone pigment (Eyal *et al.*, 1994) (Figures 1.10 and 1.11). Oosporein is considered to react with proteins and amino acids through redox reactions by altering sulph-hydryl (SH) groups, resulting in enzyme malfunction (Wilson, 1971; Strasser *et al.*, 2000). Oosporein has been described as an antiviral compound preferentially inhibiting *Herpes simplex* virus-I DNA-polymerase (Terry *et al.*, 1992) and Terry *et al.* (1992) have found it to competitively inhibit deoxyguanosine triphosphate (dGTP) or deoxycytidine triphosphate (dCTP) incorporation into DNA. Oosporein also causes avian gout in broiler chicks and turkeys and is toxic to 1-day-old male chickens (LD_{50} = 6 mg kg^{-1}) (Strasser *et al.*, 2000; Vey *et al.*, 2001). When injected intraperitoneally into mice and hamsters, oosporein was toxic at an LD_{50} value of 0.5 mg kg^{-1}. However, a daily oral administration of 7 mg kg^{-1} of oosporein to mice over a period of 47 days was non-lethal (Wainwright *et al.*, 1986). Oosporein, at 600 ng ml^{-1}, had no adverse effects against two different mammalian cell lines (Abendstein and Strasser, 2000). Oosporein at 100 µg ml^{-1} had no effect on *in vitro* cell cultures of hamster tumour cells and baby hamster kidney cells (Wainwright *et al.*, 1986). There are mixed reports of oosporein phytotoxicity: some researchers report plant growth-inhibiting and phytotoxic effects, while others report the contrary (Strasser *et al.*, 2000; Vey *et al.*, 2001).

Oosporein is said to have antibiotic properties against Gram-positive bacteria but to have little effect on Gram-negative bacteria (Vining *et al.*, 1962; Brewer *et al.*, 1984; Taniguchi *et al.*, 1984; Wainwright *et al.*, 1986). Based on published methods and results of my preliminary study, the antibiotic action of oosporein is questionable. Oosporein has also been said to have no obvious antifungal properties (Strasser *et al.*, 2000; Vey *et al.*, 2001).

1.6.5. Persistence and natural epizootics

Entomopathogenic fungal epizootics may occur sporadically in insect populations due to changes in weather, climate and host susceptibility (MacLeod *et al.*, 1966). Optimal fungal growth is in moist environments in which both the water content of the substrate and atmospheric humidity is high (MacLeod *et al.*, 1966). Conidia of many fungal pathogens generally require a minimum 70% relative humidity to germinate (MacLeod *et al.*, 1966).

Airborne dissemination of conidia is strongly influenced by external forces. If conidia are dispersed high above the ground they can be distributed randomly by air currents and may be carried great distances from their source, contingent on wind speed (MacLeod *et al.*, 1966). Conversely, if conidia are dispersed close to ground level they may not travel far. The rate at which conidia settle depends on their density, volume, surface area exposed to air, shape and atmospheric humidity (MacLeod *et al.*, 1996). Conidia will absorb water from a humid atmosphere and become heavier, thus settling more quickly (MacLeod *et al.*, 1996). Rain-washing of air may immediately terminate airborne conidial dispersion (MacLeod *et al.*, 1966).

Conidial viability can decline rapidly due to desiccation or exposure to solar (ultraviolet) radiation (MacLeod *et al.*, 1966). To increase conidial survival, delivery formulations using carriers such as oil and UV-B protectants have been investigated (Inglis *et al.*, 1995). If ambient conditions are not suitable for reproduction, fungi such as *B. bassiana* may persist as a compact mass of vegetative hyphae (i.e. a pseudosclerotium; commonly referred to as mummification) within an insect host until conditions suitable for conidiogenesis return (MacLeod *et al.*, 1966). Mummified insects may be found under bark or anchored to a substrate. Such cadavers usually disintegrate and air currents and/or rain disseminate fungal material to the soil (MacLeod *et al*., 1966). In soil, less fastidious fungi, including many of the Fungi Imperfecti, enter a saprobic phase or passive state. Persistence of *B. bassiana* while in this state is undetermined (MacLeod *et al*., 1966).

High host population density facilitates the development and spread of fungal epizootics (MacLeod *et al*., 1966) though *B. bassiana* is capable of initiating epizootics at low host population densities. Conidia can be broadly dispersed exposing the next host generation (MacLeod *et al*., 1966).

Several biotic and abiotic factors facilitating fungal epizootics must appear simultaneously for a localized outbreak to escalate to epizootic proportions (MacLeod *et al*., 1966). Such factors include solar (UV) radiation, suitable temperature, desiccation, humidity, vigour and age of infective propagules, pesticide contamination and antagonistic microbes; host behaviour, physiological condition and age are also important (Lacey and Goettel, 1995). The initiating fungus must be virulent and in sufficient density to maintain the level of infectivity and the host population must be susceptible to an infection. The moist environment created in a greenhouse cropping system is conducive to growth of both host population and entomopathogenic fungi. From the host's perspective, the greenhouse environment provides abundant nutrients, optimal abiotic conditions such as temperature for growth and space for high host population density. From the pathogen's perspective, the greenhouse provides abundant nutrients through high host population density and optimal abiotic conditions such as temperature for growth, air movement for quick dissemination and protection from solar radiation.

With knowledge of these three components (pathogen-host-environment) known as the "disease-triangle", small-scale epizootics can be triggered to control pest infestations.

1.7. Factors affecting efficacy of *Beauveria bassiana* as a biocontrol agent

1.7.1. Formulations

With prudent isolate selection, *B. bassiana* conidia can be applied against different insect pests. Most research has been directed to developing formulations for *B. bassiana* conidia (Jaronski and Goettel, 1997), with some effort to developing formulations for dry mycelial preparations using alginate or cornstarch (Pereira and Roberts, 1991). Conidial formulations used in field trials include oil-based, clay-based, wheat-bran and emulsifiable suspensions (Jaronski and Goettel, 1997). Based on field results obtained using isolate GHA conidia (Emerald BioAgriculture) and oil-based, wheat-bran, oil-clay formulations or emulsifable suspensions with/without clay, *B. bassiana* proved to be a promising control against *Melanoplus sanguinipes* (Fabricius) in North America and *Oedaleus senegalensis* (Krauss) in Africa (Jaronski and Goettel, 1997). Not all field trials have been successful, as Lobo-Lima *et al*. (1992) reported; using an oil suspension and *B. bassiana* (Emerald BioAgriculture) resulted in 30% of field-collected caged grasshoppers infected and no significant reduction in field populations (Jaronski and Goettel, 1997). Also considering that *B. bassiana* isolates are intrinsically variable, isolate selection is also a vital parameter to consider when managing different pest insects and environmental limitations (Jaronski and Goettel, 1997).

1.7.2. Beauveria bassiana use in conjunction with chemical pesticides

A possible interaction can occur when application of *B. bassiana* is integrated with that of chemical insecticides. Four documented examples demonstrate an increase in *B. bassiana* efficacy when used either with imidacloprid or dimilin. Based on a field test against *Lygus lineolaris* (Palisot de Beauvois), Steinkraus and Tugwell (1997) reported a synergistic effect between a commercial *B. bassiana* product (Mycotrol™ WP) and imidacloprid. Quintela and McCoy (1997, 1998) reported a synergistic effect between *B. bassiana* and imidacloprid applied against citrus root weevil, *Diaprepes abbreviatus* (Linnaeus). Jaronski and Goettel (1997) observed the same result when isolate GHA (Emerald BioAgriculture) was applied with dimilin (diflubenzuron) at 10% of the recommended field application rate for the control of *Melanoplus sanguinipes* (Fabricius). Thus, it is possible to use chemical insecticides with biological insecticides, thereby reducing chemical application through lower rates.

1.7.3. Beauveria bassiana use in conjunction with herbicides / fungicides

Chemical herbicides and fungicides used in greenhouses may interfere with *B. bassiana* for insect pest control. *In vitro* studies with the fungicides chlorothalonil, mancozeb, maneb, metalaxyl + mancozeb, thiophanate-methyl, zineb and the herbicide, glufosinate-ammonium, significantly inhibited *B. bassiana* mycelial growth and sporulation (Todorova *et al*., 1998). However the herbicide, diquat dibromide, could be used with *B. bassiana* as, in some cases, a synergistic effect was observed (Todorova *et al*., 1998). As with chemical insecticides, it is important to note which chemical herbicides and fungicides play an interactive role within a greenhouse.

1.7.4. Beauveria bassiana use in conjunction with other microbials

With increasing use of biological-based insecticides, it is important to consider possible interactions between such agents. There are few studies that focus on co-applying viruses, protists and bacteria with Hyphomycetes fungi to enhance efficacy (Inglis *et al.*, 2001). Wraight and Ramos (2005) reported a synergistic interaction between a commercial *B. bassiana* product (Mycotrol™ WP) and a commercial *Bacillus thuringiensis tenebrionis* product (Novodor™) applied against field populations of Colorado potato beetle larvae, *Leptinotarsa decemlineata* (Say). A suggested 'cocktail' of either 2 fungal isolates or a combination of a fungal isolate and another bioinsecticide could circumvent, biotic (e.g. interspecies antagonism) and abiotic (e.g. temperature optima), limitations thereby increasing both biological and cost efficiencies (Inglis *et al.*, 1997; 2001).

1.8. Summary

Western flower thrips are major pests in greenhouses. They cause severe damage by their feeding and reproduction; their ability to vector plant pathogens and rapidly spread and colonize. Thrips damage results in significant revenue loss to growers and pressures them to quickly take counter measures. Unfortunately, chemical insecticides are currently the primary choice for use against thrips. Exclusive use of such agents results in development of resistance and harmful effects on the environment and threats to human safety. Use of microbial insecticides is preferable due to their selectivity and minimal environmental impact. Entomopathogenic fungi, such as *B. bassiana*, are more effective than other microbials as they do not need to by ingested and offer the only microbial control option against plant sucking insects.

This chapter reviews the use of *B. bassiana* for managing western flower thrips, *F. occidentalis*. The primary focus of my thesis is to investigate the *in vitro* properties of *B. bassiana* and their relation to *in vivo* virulence. It should facilitate understanding of the dynamic interaction between *B. bassiana* and *F. occidentalis* and possibly provide a tool to rapidly screening isolates for high levels of virulence against other pest insects.

1.9. Figures

Figure 1.1. A greenhouse operation in Vancouver, British Columbia, Canada. (A) Front view with an estimated width of 128 metres. (B) Side view with an estimated length of 125 metres. An estimated total area of 16,000 m^2 (172,223 ft^2). Scale bar = 9.31 m. These and other images taken with Nikon Coolpix® 775 digital camera unless otherwise specified.

Figure 1.2. Crop production in Canadian commercial greenhouses. (A) Tomato production in Leamington, Ontario. Reservoir for biocontrol agents also shown (arrow) (B) Gerbera production in Surrey, British Columbia. Courtesy of Dr. K.M. Fry (Olds College).

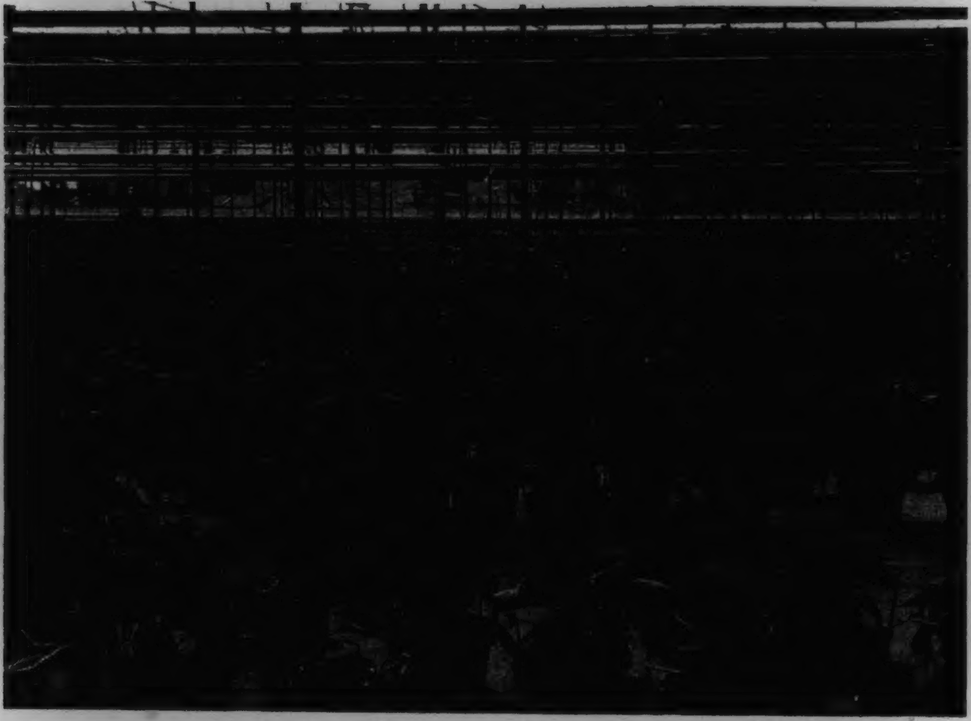

Figure 1.3. Poinsettia production at Hole's greenhouse (St. Albert, Alberta).

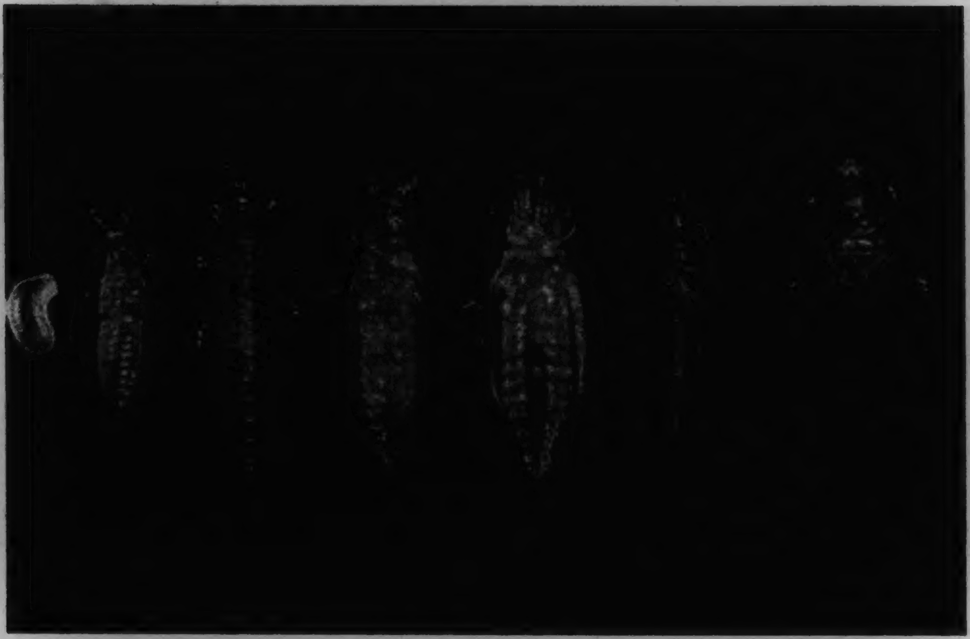

Figure 1.4. Western flower thrips, *Frankliniella occidentalis*, lifecycle. From the left: egg late in development (i.e. "red-eye" stage), first instar larva, second instar, propupa, pupa, adult male and adult female. Scale bar = 1 mm. (Clark, J.K. and Ullman, D.E. © CAB International 1997: Thrips as Crop Pests).

A
x300 0035 10kV 100µm
B
C
25µm
10µm

Figure 1.5. Adult *Frankliniella occidentalis* female genital abdominal segments and ovipositor. (A) Bi-valved ovipositor located on the 8th and 9th abdominal segment. (B) Focused image of the serrations (arrow) located on the ventral side of the ovipositor, which are used to saw through plant tissue. (C) Sensilla (arrow) appear to be campaniform mechanoreceptors. (Moritz, G. © CAB International 1997: Thrips as crop pests).

Figure 1.6. Western flower thrips *in situ* feeding damage (arrow) to cucumber fruits in a Canadian commercial greenhouse facility (Leamington, Ontario).

Figure 1.7. Western flower thrips feeding damage (arrow) to poinsettia plant leaves in a small-scale greenhouse facility (Edmonton, Alberta).

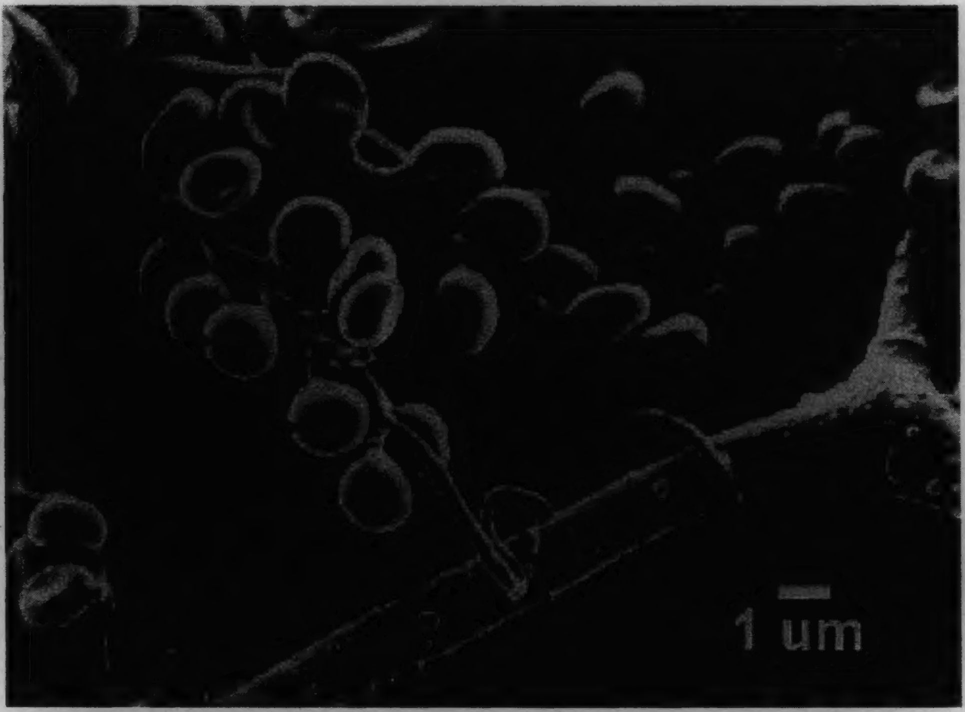

Figure 1.8. Scanning electron micrograph of *Beauveria bassiana* conidiophore and conidia. Conidia are the numerous reproductive propagules (arrow) responsible for infection. Image courtesy of Dr. B.A. Keddie.

Figure 1.9. Dead adult *Frankliniella occidentalis* female infected with *Beauveria bassiana* on a bush bean leaf. Note: Fungal re-emergence as seen with the fungal efflorescence consisting of conidiophores bearing reproductive conidia.

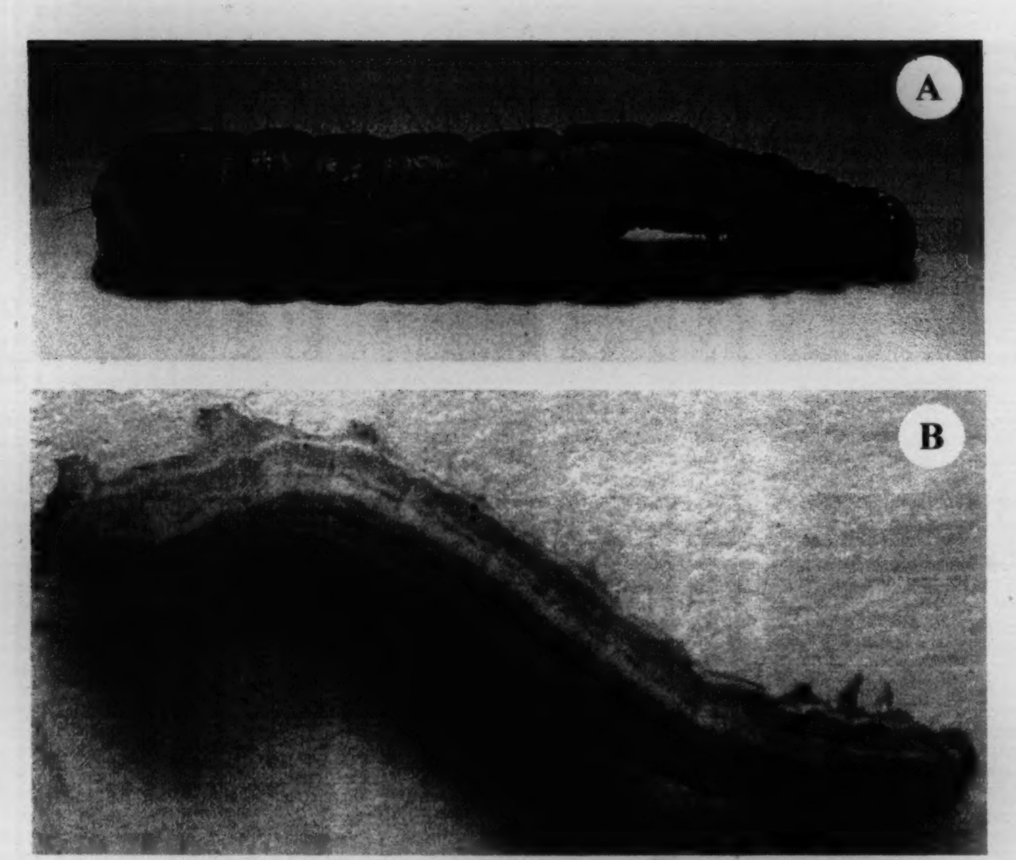

Figure 1.10. *In vivo* oosporein production in a 5th instar *Trichoplusia ni* larva. (A) Larva infected with *Beauveria bassiana* 5 days post-treatment with complete oosporein pigmentation. (B) Uninfected larva.

Figure 1.11. *In vivo* oosporein production in a 5th instar *Manduca sexta* larva. (A) Larva infected with *Beauveria bassiana* 9 days post-treatment. (B) Uninfected larva (Ex. Thomas Hörbrand-modified).

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Chapter 2. Characterization of *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) for management of western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), in greenhouse crops.

2.1. Introduction

Western flower thrips (WFT), *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), are a major pest insect of the greenhouse ornamental and vegetable industries. Thrips cause extensive damage and revenue losses through their mode of feeding and their haplodiploid (arrhenotokous) parthenogenetic reproduction (Lewis, 1997). As they feed, thrips cause direct damage to plant tissues and indirect damage through transmission of plant viruses, such as tomato spotted wilt and impatiens necrotic spot viruses (Ullman *et al.*, 1997). Due to their ability to rapidly infest, their modes of feeding and reproduction, thrips blemish crops that are undesirable to consumers. Revenue losses are proportional to damage inflicted, so growers have a low tolerance for thrips infestations.

Though chemical insecticides are currently employed to manage thrips (Murphy *et al.*, 1998; Fry *et al.*, 1999; Fry, 2003), the use of such agents may have negative consequences. For example, development of resistance leads to use of higher concentrations of active ingredients and/or to increased application with increased costs (Immaraju *et al.*, 1992; Brødsgaard, 1994; Kogel *et al.*, 1997) and to greater concern over deleterious effects on the environment and human safety (Inglis *et al.*, 2001). Thus, safer alternatives for thrips management must be developed to limit chemical use and risks to the environment and humans (Liu *et al.*, 2002).

Entomopathogenic fungi have major advantages among the strategies, both chemical and biological, currently available for controlling greenhouse pests (Sun *et al.*, 2003a). Fungal biocontrol agents are effective due to their contact route of entry, are non-toxic to higher-order non-targets organisms, can have varying degrees of host specificity and can be mass cultured *in vitro* which reduces costs (Sun *et al.*, 2003a). They can be used where chemical pesticides are banned (e.g. some organochlorines) or are being phased out or where pests have developed resistance to conventional pesticides (Butt *et al.*, 2001). Specifically, *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) has been shown to be a promising alternative to chemical control for western flower thrips (Murphy *et al.*, 1998; Fry *et al.*, 1999; Fry, 2003). Moreover, *B. bassiana* has been described as a model organism for the study of entomopathogenesis and the biological control of pest insects (Rehner and Buckley, 2005).

Entomogenous fungi belonging to the genus *Beauveria* produce a red-coloured dibenzoquinone pigment known as oosporein (Eyal *et al.*, 1994; Strasser *et al.*, 2000; Vey *et al.*, 2001) (Figure. 2.1). Oosporein is known to react adversely with sulph-hydryl (SH) groups of amino acids that are components of enzymes (Wilson, 1971). Oosporein is said to have antibiotic properties on Gram-positive bacteria but have little effect on Gram-negative bacteria (Vining *et al.*, 1962; Brewer *et al.*, 1984; Taniguchi *et al.*, 1984; Wainwright *et al.*, 1986). Based on preliminary study I believe that oosporein may instead have a novel role in the lifecycle of *B. bassiana* and possibly in those of other oosporein-producing fungi.

Beauveria bassiana is a cosmopolitan entomopathogen and although mitosporic; variations are known within and among isolates (St. Leger *et al.*, 1992; Maurer *et al.*, 1997; Castrillo and Brooks, 1998; Castrillo *et al.*, 1999). Genetic recombination within

B. bassiana is primarily a result of the parasexual cycle whereby the presence of two different types of nuclei in the mycelium (heterokaryosis) may result in fusion to produce an unstable diploid nucleus (Pontecorvo, 1956; Alexopoulos *et al.*, 1996). This condition can occur by mutation or by fusion of mycelia with different types of nuclei (Pontecorvo, 1956; Alexopoulos *et al.*, 1996). Diploid nuclei can spontaneously generate diploid recombinants by mitotic crossing over and/or haploid recombinants by non-disjunction, both events occurring at low frequencies (Pontecorvo, 1956; Paccola-Meirelles and Azevedo, 1991; Alexopoulos *et al.*, 1996). This genetic variability among isolates of *B. bassiana*, translates into varying degrees of virulence towards a target insect. Even though *B. bassiana*, in general, exhibits a broad host range, individual isolates exhibit various degrees of host specificity (Goettel, 1992). As a result, individual isolates of *B. bassiana* are routinely screened against a target insect for efficacy and virulence.

In vivo studies using whole animals are widely conducted to evaluate entomopathogenic fungi for efficacy and virulence. Although whole insect bioassays provide useful information on these characteristics, such tests are laborious and time consuming (Fornelli *et al.*, 2004). Another approach is to screen fungal isolates based on their *in vitro* growth characteristics.

Previous studies have shown that physiological characteristics and enzyme production of fungi relate to their virulence (Paris and Ferron, 1979; Paris *et al.*, 1985; Samuels *et al.*, 1989; Bidochka and Khachatourians, 1990; Feng and Johnson, 1990; Liu *et al.*, 2003). Liu *et al.* (2003) demonstrated other characteristics such as conidial viability, speed of germination, hyphal growth rate, infectivity and spore production in response to environmental temperature; relative humidity and ultraviolet (UV) light also influences efficacy of fungal isolates as microbial control agents.

In this study, *B. bassiana* isolates, previously evaluated for virulence against western flower thrips (Litwinowich, 2005), are further evaluated for their *in vitro* growth characteristics to assist in predicting whole animal virulence and for rapidly screening *B. bassiana* isolates with high virulence. Median lethal time to kill 50% of the thrips population (LT_{50}) is used as a measurement of virulence. Median lethal times are a more suitable measure for comparing *in vitro* growth characteristics. Median lethal concentration required to kill 50% of the thrips population (LC_{50}) also provides information about isolate virulence by quantifying mortality; however median lethal time addresses rate of infection, a measure of fungal growth rate within a host. Virulence parameters such as LC_{50} and LT_{50} pinpoint different aspects of the infection process but the time parameter relates more effectively to *in vitro* characteristics of a fungal isolate.

To my knowledge, few studies have been conducted to assess *in vitro* growth characteristics and to correlate them with whole animal virulence. Here I describe a novel approach to assess *in vitro* growth characteristics. I also discuss the potential role of oosporein in the lifecycle of *B. bassiana* with photo-activation based on preliminary results using solid and liquid media (Figure 2.2).

2.2. Materials and methods

2.2.1. *Western flower thrips*

Specimens of *Frankliniella occidentalis* (Pergande) were acquired from a colony maintained at the Alberta Research Council (Vegreville) and a colony was established in our laboratory in 2002. As described by Litwinowich (2005), thrips were reared in 1.89-litre containers (Gladware®, The Glad Products Company, Oakland, California) at 25°C

± 1°C and 16:8 h (light:dark). Containers were modified for air circulation by cutting a 3 x 15 cm rectangular opening and covering the opening with 80 µm (mesh pore size) Nitex® nylon fabric (Sefar America Incorporated, Depew, New York) glued in place with clear silicone (GE silicone I) rubber sealant. Each chamber was lined with one 4 x 17 cm piece of absorbent non-sterile cotton (Padco Corporation, Washington, DC), followed by two 11 x 17 cm pieces, the top piece wrapped in a layer of cheesecloth providing a supportive matrix. Each rearing container received 400 ml of sterile deionized water (Continental Modulab® system, Continental Water Systems, San Antonio, Texas, model# LBPUU101002) to maintain adequate moisture levels. Relatively uniform 19-day old unblemished bush bean leaves, *Phaseolus vulgaris* Linnaeus (Mackenzie Tendergreen™ Improved bush bean seeds, McKenzie Seeds, Brandon, Manitoba), were cut leaving each with a 3 cm petiole. The petiole of each leaf was inserted into a dimple drilled in the cheesecloth/cotton matrix of each arena. The cotton was then packed around the petiole. Each leaf was dusted once with non-viable apple pollen (Antles Pollen Supplies, Wenatchee, Washington), covering 2 x 2 cm in the central area of the leaf to serve as a protein source for the thrips (Figure 2.3). Once the rearing chambers were established, 35-50 adult female thrips from a 21-day old colony were added to each chamber for mating and oviposition. Seventy-two hour post incubation, all adult thrips were removed and two new, large, 22-day old bush bean leaves were placed one above and one below the newly impregnated original leaf for emerging larvae. Rearing chambers were incubated in a growth chamber (Sanyo Scientific, Concord, Ontario, model# MLR-350) at the conditions specified above for 18 days until adults had developed at which time they were used for whole animal bioassays; at 21 days, remaining adults were used to maintain the laboratory colony.

2.2.2. Beauveria bassiana solid medium

Each isolate was cultured on half-strength (0.5x) Sabouraud dextrose agar (SDA) [BD (Difco™)/BBL, Oakville, Ontario)] (1.5% final agar concentration) supplemented with 0.5% yeast extract (YE) [BD (Difco™), Oakville, Ontario] (0.5xSDAY) (Goettel and Inglis, 1997; Goettel, personal communication). Antibiotics were not incorporated for routine culturing, as such may place an unnatural stress on fungal isolates being cultured and may elicit a secondary response in them (Currah, personal communication).

2.2.2.1. Media preparation

The lot number and date of receipt of each container of SDA was recorded and a unique identifier assigned which was used to track each batch preparation throughout the study. A sample from each lot was also collected and stored in a sealed sterile 15 or 50 ml centrifuge tubes at room temperature (23°C ± 2°C) for future analysis. A record was kept of each preparation for quality control.

All measuring instruments were cleaned with 70% ethanol to eliminate residue contamination. Erlenmeyer flasks used for media preparations were at least double the desired media volume.

Dehydrated media was added to each Erlenmeyer flask along with a stir bar cleaned with 70% ethanol and 1000 ml of deionized water was added. The flask was sealed with a slitted aluminum foil cap and autoclave paper secured with a heavy rubber band. The flask was heated on a hot plate to boiling for 1 min with constant stirring.

After boiling, the medium was autoclaved at 121°C/15 pounds per square inch (psi) for 17 min.

Immediately after autoclaving, medium was poured into plastic petri plates (Fisherbrand®, Fisher Scientific, Ottawa, Ontario) within a biosafety cabinet to prevent contamination. Successive plates were stacked seven high to reduce condensation and to increase pouring efficiency. Plates remained under UV light for a minimum of 60 min while the agar annealed.

Once the agar annealed, plates were sealed back in their original sleeves inside the biosafety cabinet. Each sleeve of plates was dated and labeled. Plates were stored briefly at room temperature (23°C ± 2°C) to check for contamination and to reduce condensation. Plates were stored at 4°C or left at room temperature if they were to be used in the near future.

One litre of medium yielded approximately 50 100 x 15 mm petri plates or 100 60 x 15 mm petri plates. The amount of medium added into each petri plate was optimized for conidial production (Kamp and Bidochka, 2002) and cost efficiency. Media was prepared approximately every 14 days to ensure a continuous supply of fresh plates.

2.2.3. Beauveria bassiana isolates

Twenty *B. bassiana* isolates were included in this study (Table 2.1). Isolates were selected from various hosts although few exhibited signs of disease. Isolates were recovered from 8 host species collected in the greenhouse and field. Most isolates were obtained from Dr. Matt Greif (University of Alberta, Edmonton, Alberta).

Isolate GHA, obtained from Dr. Ken Fry (Olds College, Olds, Alberta; formerly at Alberta Research Council, Vegreville, Alberta)/ Dr. Mark Goettel (Agriculture and Agri-Food Canada, Lethbridge, Alberta), was originally acquired from Emerald BioAgriculture (Lansing, Michigan) (formerly Mycotech Corporation, Butte, Montana) as unformulated dry conidia and used as an industry standard. When received, this isolate in both unformulated and a formulated commercial product (BotaniGard™ ES, emulsifiable suspension) was contaminated with an *Enterobacter* bacterium common to both products with 97% 16S rRNA similarity to *Enterobacter cloacae* (Rajput and Zurek, unpublished data). This bacterial contaminant appears to be consistently associated with isolate GHA, as other laboratories have also encountered this contaminant (James *et al.*, 2003) including another formulated commercial product (BotaniGard™ 22WP, wettable powder) (Labbe, personal communication). Based on preliminary study, this contaminant appears to increase the efficacy of isolate GHA by causing a secondary infection of the host. This observation is consistent with the reduced mortality recorded by James *et al.* (2003). James *et al.* (2003) may have removed the contaminant before subsequent assays with isolate GHA by passaging their isolate GHA through silverleaf whitefly nymphs prior to re-isolating on solid medium. All tests reported here used isolate GHA free of detectable contaminants as established by use of standard subculturing and dilution plating techniques.

Isolates were subcultured on 0.5xSDAY and incubated in the dark at 23°C ± 2°C for 14 days. To prevent possible attenuation of virulence, a series of subcultures were prepared from these plates and held at 4°C as described by Liu *et al.* (2003). To ensure viability and preserve virulence, cultures were stored at 4°C under sterile deionized water stasis, lypholized or as dry conidial spores (Humber, 1997; López Lastra *et al.*, 2002). These purified and minimally subcultured isolates were used in all tests conducted in this study.

All isolates were identified as *Beauveria* spp. using Hoog's (1972) description of gross colony morphology and by microscopy based on the descriptions of conidiophores and conidia of MacLeod (1954) and Hoog (1972).

2.2.3.1. Beauveria bassiana inoculum preparations

To prepare *B. bassiana* inoculum, conidia from each isolate subcultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days were harvested using a sterilized bacteriological loop and were suspended in 2000 µl of sterile deionized water adjusted to a concentration of 10^6 spores/ml.

2.2.3.2. Beauveria bassiana conidia production

For mass production or amplification of selected isolates, previously described procedures (Goettel and Inglis, 1997; Fry, 2003) were implemented with minor modifications. With each isolate, 100 µl of suspended spores was plated onto each of ten 100 x 15 mm 0.5xSDAY plates and spread using a sterile glass spreader. Each plate was individually sealed with parafilm and incubated in a dark chamber at 23°C ± 2°C for 14 days. Each chamber consisted of an inverted 28 cm x 23 cm x 43 cm copy paper cardboard box fitted with a lid and lined with black construction paper (Figure 2.4). This prevented light penetration, measured at zero foot-candles with a Gossen hand-held light meter (Nürnberg, Germany).

2.2.3.3. Beauveria bassiana drying procedure

Fourteen days post incubation mature cultures were removed from the chamber(s) and visually inspected to ensure that growth of each isolate was consistent throughout the set of 10 plates and that no visible contamination was present either on the medium surface or between the lid and base of the petri plate. Prior to placement in a drying chamber, parafilm was removed sequentially from each petri plate until all the plates of each isolate were completed. Each set of plates was placed under an inverted, Reynolds® aluminum-roasting pan (43.0 cm x 31.7 cm x 6.7 cm), set on an aluminum foil-covered lab bench (Figure 2.5). Plates were evenly spaced, with the drying chamber opened slowly at ~45° for only brief period while loading. Once all the plates were in place, each lid was partially opened. Before handling the next isolate set, gloved hands were thoroughly sprayed with 70% ethanol to prevent any cross-contamination. These steps were repeated for all isolates.

If an isolate exhibited characteristics of powder-like conidia (i.e. most to complete hyphal degradation), these were set for drying last to minimize the risk of cross contamination. Based on my observations, these isolates appear to have stronger hydrophobic properties and retain less moisture than other isolates and thus are likely more sensitive to static electricity creating an increased risk for cross-contamination.

Isolates were harvested after 48 h of passive drying, that is, no active air circulation. This contrasts with the system used by Fry (2003), whereby cultures were placed within a biosafety cabinet with constant air circulation (i.e. active).

The drying chambers were sprayed with 70% ethanol after an isolate was dried and removed.

2.2.3.4. Beauveria bassiana harvesting procedure

Within the drying chamber, lids of each plate were closed and the set of 10 plates were transferred to a sterilized biosafety cabinet. Cultures were harvested by scraping at a ~45° angle into a sterile 60 x 15 mm petri plate with a sterilized rubber policeman attached to a 1 ml borosilicate pipette. Slow and steady strokes were used to minimize aerosol formation and allowed for consistent *in vitro* morphological assessments. Subsequent to completing an isolate set, the harvested plates were set off to the side and the single 60 x 15 mm receiving petri plate was sealed with parafilm and placed into a 10 x 12 cm polyethylene bag (Fisher Scientific, Ottawa, Ontario). The bag was folded into a square and secured with two heavy rubber bands (Grand & Toy, Don Mills, Ontario) at right angles to each other. All preparations were stored at 4°C under desiccation (Figure 2.6). To measure aerosol dispersion within the safety cabinet, one 100 x 15 mm plate of 0.5xSDAY was placed at the rear left-hand corner of the biosafety cabinet fully exposed during the harvest of each isolate. The plate was sealed with parafilm and incubated at

23°C ± 2°C. No growth was observed on these plates throughout the recovery of all isolates.

When an isolate series was harvested the following procedure was followed. All materials were removed from the biosafety cabinet and all surfaces were treated with 70% ethanol, followed by 10% household bleach solution (0.525% sodium hypochlorite), wiped down with sterile tissues (KimWipes®, Kimberly-Clark, Mississauga, Ontario) and then 70% ethanol with a final wipe with sterile tissues (KimWipes®, Kimberly-Clark, Mississauga, Ontario). After this procedure was complete, all the materials required for the next isolate harvest were placed within the cabinet. Fungal growth characteristics were recorded after each isolate harvest.

This procedure was carried out for pooled screening trials (n = 7) consisting of 19 isolates and for pooled completely replicated evaluation trials (n = 3) consisting of 5 isolates for a total of 20 isolates used in this study including isolate GHA.

2.2.3.4.1. Beauveria bassiana characteristics

2.2.3.4.1.1. In vitro morphological characteristics

To provide a measure of whole animal virulence, six *in vitro* morphological characteristics were assessed.

1. *Harvest quality.* This was based on conidial production and ease of harvest, ranging from: poor (1), moderate (2), good (3), and excellent (4). This characteristic can be categorized on a broad scale for easy isolate reference.

2. *Tendency for mycelium to fragment and form tufts of mycelial fragments during harvest of each isolate*. Mycelial fragments were described as thin (1) (Figure 2.7) or thick (2) (Figure 2.8). If no mycelial fragments remained (i.e. complete mycelial degradation), then this would be described as powder-like conidia production (3) (Figure 2.9). This mycelial-based characteristic was apparent during the harvest of over 20 different isolates. Both thin and thick mycelia fragments produced conidia though thin fragments yielded significantly more conidia than thick fragments based on spore counts using a Reichert Neubauer Improved Bright-Line haemocytometer (Reichert Incorporated, Depew, New York).

3. *Degree of submerged mycelia ("rooting")*. This characteristic was defined based on degree of mycelial tenacity (i.e. difficulty in removing mycelia from the agar surface), ranging from: none (1), moderately submerged (2), to deeply submerged (3). If an isolate exhibited no mycelial penetration within the agar (i.e. growth restricted to agar surface), it was easy to harvest and yielded a substantial amount of fungal material, this would be described as none (1). If isolate mycelia were partially submerged within the agar but could be harvested easily (i.e. loosely attached) and yielded a substantial amount of mycelial fragments/conidia, this would be described as moderately submerged (2). If isolate mycelia were deeply submerged and adhered tightly to the agar and thus difficult to harvest and yielded little fungal material, this would be described as deeply submerged (3).

4. *Aerial mycelia or elevation ("rise")*. Mycelial elevation was characterized ranging from: low (~flat) (1), mid (above the agar surface to midway up the height of the petri dish) (2) to high (from the midpoint of the petri dish to the base of the lid) (3). This characteristic likely reflected the metabolic rate of the isolate. Due to the standardized

complexity of 0.5xSDAY medium, the mycelial elevation of an isolate suggested the rate at which an isolate depleted available nutrients and switched from vegetative (i.e. mycelia) to reproductive state. Isolates with a putative high metabolic rate, will deplete available nutrients quickly and shift to producing reproductive structures with relatively minimal vegetative growth or rapid mycelial degradation would be induced, yielding low-rising (~flat) cultures. Isolates with a putative low metabolic rate, will deplete available nutrients slowly and remain primarily in the vegetative state for most of the 14-day incubation period, yielding high-rising cultures (sometimes mycelial growth is observed from the agar surface to lid of the petri dish). Isolates with a putative intermediate metabolic rate, will shift at some point midway within the 14-day incubation period to the reproductive state, yielding mid-rising cultures.

5. *Yellow mycelial pigmentation*. This yellow colouration may perhaps be due to oxalic acid production. Isolates were categorized as absent (white) (0) or present (1).

6. *Oosporein production was based on red pigmentation*. Isolates were categorized as absent (0) or present (1).

2.2.3.4.1.2. Quantitative characteristics

2.2.3.4.1.2.1. Conidial viability

Viability assessments were conducted using an approach modified after Firstencil *et al*. (1990). Stock solutions of propidium iodide (Sigma Chemical, St. Louis, Missouri) consisting of 0.3% (weight/volume) using sterile deionized water were prepared every 6 months. Immediately prior to each test, a working stock consisting of 1.2%

(volume/volume) stock propidium iodide solution diluted in sterile deionized water was prepared.

Conidia were suspended in 500 μl of sterile 0.01 M sodium phosphate buffer (pH 7.0) containing 0.01% Tween-80 (Sigma Chemical, St. Louis, Missouri) adjusted to a concentration of 10^7 conidia/ml for each test. After thorough vortexing, 7.0 μl of conidia suspension was mixed with an equal volume of propidium iodide working stock on a pre-cleaned microscope slide (Fisherbrand®, Fisher Scientific, Ottawa, Ontario) and covered with a 22 x 22 mm coverslip. Conidial and vital dye preparation was conducted within a dark room under a red safe light. Each sample was transported in a sealed dark container to an epifluorescence Reichert-Jung® Polyvar microscope (Kandel Electronics Incorporated, Oreland, Pennsylvania). Each treated conidial suspensions was viewed by fluorescence microscopy under 400x magnification. A minimum of 1000 conidia were scored in four randomly selected fields of view. Conidia fluorescing bright red were scored as nonviable and those remaining dark with a red halo were scored as viable (Figure 2.10). Percent viability was calculated by dividing the number of viable conidia by the total number of conidia counted, multiplied by 100. Fungal viability assessed in this manner was corroborated by comparisons with fluorescein diacetate (a vital dye used to indicate viable conidia) (Sigma Chemical, St. Louis, Missouri) fluorescence microscopy (Firstencil *et al*., 1990) and plate counts (Goettel and Inglis, 1997; Rajput and Litwinowich, unpublished data).

Fluorescence microscopy allowed for rapid, accurate and precise assessment of isolate viability. Once fluorescence reagents were prepared, viability assessments required about 20 min per isolate whereas plating required days to assess isolate viability.

This method avoided inaccurate plate counts that can result from observing conidial swelling due to damaged cell membranes of non-viable cells and scoring these as viable.

2.2.3.4.1.2.2. *Whole animal virulence*

Twenty isolates of *B. bassiana* used in this study were selected based on their virulence (LT_{50}) against western flower thrips (Litwinowich, 2005) (Tables 2.2 – 2.4). Adult female thrips were indirectly exposed to four conidial concentrations of each isolate of *B. bassiana* by spraying a bush bean leaf (*P. vulgaris*) disc. Exposed insects were monitored for mortality for 5 days post-spray excluding day 1. Mortality within 24 h was attributed to treatment trauma. Mycosis was confirmed by plating dead insects on solid selective 0.055% dodine/0.0005% tetracycline medium (Chase *et al.*, 1986) and incubating plates at 23°C ± 2°C until fungal emergence.

2.2.3.5. *Beauveria bassiana statistical analyses*

All statistical analyses were performed using SAS software version 8.2 (SAS Institute, Cary, North Carolina). Based on the characteristics above, 4 categories were designed for statistical analyses: Category 1 ("growth"): harvest quality, mycelial fragments and degree of submerged mycelia; category 2 ("aerial"): aerial mycelia; category 3 ("yellow"): yellow colouration of mycelia; and category 4 ("red"): red (oosporein) colony/medium colouration. Since category 1 consisted of 3 combined characteristics, a numerical coding matrix was designed to integrate all 3 characteristics. This was done by coding category 1 ("growth") from 1 to 36 corresponding to the sequential codes of the respective characteristics. For example, if category 1 was coded as 1, this would refer to an isolate with harvest quality (1), mycelial fragments (1) and degree of submerged mycelia (1) (Table 2.5). This category was created because, individually, the three characteristics were not statistically significant. Sun *et al*. (2003b) also combined characteristics in their work to determine their relation to virulence. My system of coding is more accurate than Sun *et al*. (2003b) in accounting for variability as they simply grouped isolates in one of two categories (each consisting of 3 characteristics), which can draw attention away from the overall inherent variability observed in practice.

Isolates yielding inconsistent growth or whole animal virulence characteristics were removed from analysis.

Growth morphological categories were subjected to Analysis of Variance (ANOVA) using a Generalized Linear Model (GLM) procedure. The primary purpose of doing so was to see if Categories (1), (2), (3) and (4) were significantly different by comparing the dependent variable of whole animal virulence (LT_{50}). This allowed one to determine if any characteristics can be applied to predict *in vivo* virulence using *in vitro* assessments. Screening trials and evaluation trials respectively were pooled based on the statistical analyses of Litwinowich (2005) indicating non-significance. Differences were considered significant at $p < 0.05$.

2.2.4. Beauveria bassiana in vitro oosporein production

2.2.4.1. Beauveria bassiana broth medium

Each isolate was cultured in 150 ml of full-strength (1x) Sabouraud dextrose broth (SDB) [BD (Difco™)/BBL, Oakville, Ontario)] supplemented with 1% yeast extract (YE) [BD (Difco), Oakville, Ontario] (1xSDBY) (Goettel and Inglis, 1997). Full strength medium was used due to its complexity in providing a full range of undefined growth factors (Madigan *et al.*, 1997).

2.2.4.2. Beauveria bassiana isolates

Four isolates including isolate GHA were selected for further investigation with reference to oosporein production based on whole animal virulence data (ranged from low to high performance) (Table 2.6).

2.2.4.2.1. Beauveria bassiana inoculum preparations

B. bassiana inocula were prepared by selecting isolates subcultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days, harvesting them using a sterilized bacteriological loop and suspending them in 2000 µl of sterile deionized water adjusted to a concentration of 10^6 spores/ml.

2.2.4.2.2. Beauveria bassiana liquid cultures

Four sterile 250 ml Erlenmeyer flasks filled with 150 ml of 1xSDBY were each inoculated with 1000 µl of spore suspension beginning with isolate GHA. An additional, non-inoculated, flask filled with 150 ml of medium was prepared as a negative control. Isolate SR-25 was included based on its high virulence and due to its consistent oosporein production during solid media culturing thus acting as a positive control.

Inoculated flasks were incubated at 25°C ± 2°C, 16:8 h (light:dark) within an upright incubator with 2 cool-white fluorescent bulbs (Philips F32T8/TL741 bulbs, Philips Canada, Markham, Ontario). Flasks were positioned at equal distances from the bulbs to ensure even exposure. Light intensity was measured to be 220-290 foot-candles at the flasks position using a Gossen hand-held light meter (Nürnberg, Germany).

Cultures were monitored daily for oosporein production for 14 days.

2.3. Results

2.3.1. Collection and identification of isolates of Beauveria bassiana

Based on microscopic examination and culture morphology, all 20 isolates were determined to be *B. bassiana*. Sympodial to whorled conidial clusters are presented in Figure 2.11.

Culture development of every isolate of *B. bassiana* was consistent with Hoog's (1972) colony description of the type species for the genus:

> "*Beauveria* colonies growing moderately slowly, appearing lanose, powdery or funiculose, rarely forming synnemata, white or yellowish, occasionally pinkish. Aerial hyphae hyaline, smooth- and thin-walled, loose or sometime fasciculate."

Microscopic examination of all isolates was also consistent with Hoog's (1972) and Rehner and Buckley's (2005) descriptions of *B. bassiana*:

> "*Beauveria bassiana* is characterized morphologically by its sympodial to whorled clusters of short-globose to flask-shaped conidiogenous cells, which give rise to a succession of one-celled, hyaline, holoblastic conidia that are borne on a progressively elongating sympodial [denticulate] rachis."

2.3.2. Beauveria bassiana characteristics

Twenty isolates of *B. bassiana* ranging from low to high levels of whole animal virulence (LT_{50}), were selected for further *in vitro* morphological analyses of growth to determine whether this analysis could provide a more efficient isolate screening system (Figures 2.12 – 2.24).

Pooled screening (n = 7) and evaluation trials (n = 3) showed category "growth" to be statistically significant but not the categories "aerial", "yellow" and "red" (Table 2.7).

Viability of the isolates used within pooled screening trials ("trial 1") and each replicate evaluation trial are shown in Tables 2.8 and 2.9. Within pooled screening trials ("trial 1") isolates had a general viability ranging from 85.0% - 99.8% with the exception of isolate SR-7 with a low viability of 45.3%. Across evaluation trials, isolates generally had a viability ranging from 88.9% - 100.0% (Table 2.9). All final conidial concentrations were corrected for viability prior to use within thrips bioassays (Litwinowich, 2005).

2.3.3. Beauveria bassiana in vitro oosporein production

Four isolates of *B. bassiana*, ranging from low to high levels of whole animal virulence and/or *in vitro* growth characteristics, were selected for additional metabolite analysis. Oosporein production using light as an induction factor was examined visually every 24 h. Growth was evident as early as 48 h post inoculation (Table 2.10).

All *B. bassiana* isolates cultured *in vitro* using 1xSDBY liquid medium, produced oosporein as early as 48 h post inoculation (isolate SR-35) and as late as 144 h post inoculation (isolate SR-11) (Table 2.11; Figures 2.25 – 2.29).

2.4. Discussion

2.4.1. Beauveria bassiana characteristics and their relation to whole animal virulence

To evaluate entomopathogenic fungi for increased efficacy or high levels of virulence, *in vivo* studies using whole animals are conducted. Although, whole insect bioassays provide useful information on toxicity towards a target organism, such tests are laborious and time consuming (Fornelli *et al*., 2004). Another approach is to screen fungal isolates based on their *in vitro* growth characteristics.

In this study, *B. bassiana* isolates previously bioassayed against western flower thrips (Litwinowich, 2005) were evaluated for their *in vitro* growth characteristics. This data was analyzed to determine whether one or a combination of these characteristics could be used to predict whole animal virulence. I used median lethal time to kill 50% of the thrips population (LT_{50}) as a measure of whole animal virulence and the dependent variable in addressing the question of *in vitro* growth characteristics versus virulence. Median lethal time addresses rate and is conceivably coupled with the metabolic characteristics of a fungal isolate thus linking it to its *in vitro* growth characteristics.

To address this question, I scraped plates with a rubber policeman and assessed characteristics of mycelial and conidial growth. *In vitro* growth morphologies were assessed based on: harvest quality, mycelial fragments (if present), degree of submerged mycelia ("rooting"), aerial mycelia or elevation ("rise"), mycelial pigmentation (specifically yellow colouration) and red (oosporein) colony/medium pigmentation. Morphological growth characteristics were assigned to one of four categories. These were then subjected to statistical analyses based on LT_{50} data previously collected from western flower thrips bioassays. Screening and evaluation trials were both pooled based on the statistical analyses of Litwinowich (2005) indicating non-significance within and among trials.

For pooled screening (n = 7) and evaluation trials (n = 3), category "growth" was statistically significant (screen trial: $p > 0.0324$, $\alpha = 0.05$; evaluation trial: $p > 0.0179$, $\alpha = 0.05$) (Table 2.7). This indicated that characteristics within the category "growth" could be used to predict *in vivo* virulence. I believe that isolates of *B. bassiana* exhibiting good/excellent harvest quality, thin or very few (i.e. powder-like conidia) mycelial fragments and a none/moderate degree of submerged mycelia will have a high probability of having high *in vivo* virulence. Isolates of *B. bassiana* exhibiting poor/moderate harvest quality, thick mycelial fragments and a high degree of submerged mycelia will have a high probability of having low *in vivo* virulence. However, it is important that isolates exhibit generally consistent growth morphologies for accurate *in vivo* virulence predictions to be made and exceptions are possible.

Category "aerial" was statistically insignificant for both pooled screening and evaluation trials (screen trial: $p > 0.9244$, $\alpha = 0.05$; evaluation trial: $p > 0.3531$, $\alpha = 0.05$) (Table 2.7). In my preliminary studies using 1xSDAY medium, aerial mycelial growth of *B. bassiana* did provide some information on the metabolic rate of the isolate as to how rapidly it would shift from vegetative to reproductive growth. For example, if an isolate had a relatively slow metabolic rate, it would remain in the vegetative state longer than an isolate with a relatively higher metabolic rate and therefore would exhibit higher aerial mycelial growth. In most cases, isolates that maintained vegetative growth suggested a relatively slow metabolism and low *in vivo* virulence. As I optimized the medium for maximum conidial production, by lowering the strength (i.e. 0.5xSDAY) and medium depth poured, the resolution of this characteristic became unclear. Subsequent to day 16 of culturing/drying, most isolates had low-mid elevation resulting in non-significance, as expected.

Neither was category "yellow" significant in either pooled screening or evaluation trials (screen trial: $p > 0.2160$, $\alpha = 0.05$; evaluation trial: $p > 0.3531$, $\alpha = 0.05$) (Table 2.7). This indicated that yellow mycelial colouration, due to possible oxalic acid production, was not associated with *in vivo* virulence and thus, cannot be used as an indicator. This result was confirmed by Bidochka and Khachatourians (1993) who reported that oxalic acid is not related to virulence in grasshoppers, *Melanoplus sanguinipes* (Fabricius) but to a utilizable carbon source.

Category "red" was statistically insignificant for both pooled screening and evaluation trials (screen trial: $p > 0.6236$, $\alpha = 0.05$; evaluation trial: $p > 0.6246$, $\alpha = 0.05$) (Table 2.7). This clearly indicated that red pigmentation caused by oosporein production was not associated with *in vivo* virulence and thus, could not be used as an indicator.

Tables 2.8 and 2.9 indicate the viability of isolates used within the pooled screening trials ("trial 1") and in each replicate evaluation trial. In the pooled screening trials ("trial 1"), isolates had viabilities ranging from 85.0% - 99.8% with the exception of isolate SR-7 (45.3%). Across evaluation trials, isolates had viabilities ranging from 88.9% - 100.0%. All final spore concentrations were corrected for viability prior to use within thrips bioassays (Litwinowich, 2005). Consistently high viabilities confirmed that the production and processing methods were suitable for *B. bassiana*. No statistical analyses were performed on viability data, as consistently high viability would yield non-significance.

To my knowledge, results of only one other study concur with mine. Sun *et al.* (2003b) examined *in vitro* morphological growth characteristics of *B. bassiana* and *Metarhizium anisopliae* (Metschnikoff) Sorokin isolates to determine if there was any correlation to termite (*Coptotermes formosanus* Shiraki) virulence using LD_{50} data. They concluded that growth characteristics correlated significantly with virulence against termites, suggesting that characteristics of a fungus growing on agar might contribute to estimating fungal virulence *in vivo*. I agree with their concluding statement. However, the *in vitro* characteristics used to assess isolates and their methods of categorizing isolates are questionable. Sun *et al.* (2003b) categorized both fungi based on *in vitro* growth into two groups: (1) colonies with compact growth, flat elevation and >50% of the surface area sporulating after 12 days of growth; and (2) colonies with filamentous growth, raised elevation and <50% sporulation according to criteria of Hawksworth (1974) and Klein (1996). They then performed statistical analyses (i.e. ANOVA) on the differences in LD_{50} data. Sun *et al.* (2003b) did not provide the precise meanings of the characteristics employed. In reviewing Hawksworth (1974) and Klein (1996) further clarity on the meanings were not found. The characteristics used by Sun *et al.* (2003b) are obscure and are difficult to extend to the results of other researchers. Based on my interpretation of the observations of Sun *et al.* (2003b) and my experience with *B. bassiana*, I suggest that at least another category should have been included in their descriptions which includes colonies with filamentous growth, raised elevation and >50% sporulation. Broadly categorizing fungal isolates into two categories each consisting of three characteristics, as they did, is not as accurate as the statistical matrix table used in my study: intrinsic variation naturally existing in many fungi including *B. bassiana*, is not considered with Sun *et al.*'s (2003b) methods. My statistical matrix table allows for natural variation and maintains a clear separation of isolates based on their level of virulence. Sun *et al.* (2003b) also categorized two different fungal species according to one set of characteristics. One set of characteristics cannot be accurately transferred to two fungal species even though they both belong to the same phylum. This may explain the lack of clarity in their descriptions.

Other approaches may be used to compare *in vitro* characteristics to whole animal virulence as a means to selecting isolates with increase efficiency. Sun *et al.* (2002) compared *in vitro* versus *in vivo* conidial production for *B. bassiana* and *M. anisopliae* to select effective isolates for control of the termite *C. formosanus*. Sun *et al.* (2003a) also characterized isolates of *B. bassiana* and *M. anisopliae* based on their rate of and total sporulation in relation to virulence. Liu *et al.* (2003) examined conidial size, viability, spore production, germination rate, relative hyphal growth and temperature sensitivity of *B. bassiana* and *M. anisopliae* isolates to select isolates to control the lygus bug, *Lygus lineolaris* (Palisot de Beauvois). Although these studies provide useful information on potential *in vivo* virulence, consideration of mycelial growth in its relation to its growth state (i.e. vegetative or reproductive), its degree of submergence within solid agar and its aerial growth (depending on media used) is also worth examining.

2.4.2. *A potential role for oosporein in the life history of Beauveria bassiana*

Many soil and entomogenous fungi in the genus *Beauveria* produce a red-coloured dibenzoquinone pigment known as oosporein (Eyal *et al.*, 1994; Strasser *et al.*, 2000; Vey *et al.*, 2001) (Figure 2.1). Oosporein has been constantly cited as an effective antibiotic against Gram-positive but to have little effect on Gram-negative bacteria (Vining *et al.*, 1962; Brewer *et al.*, 1984; Taniguchi *et al.*, 1984; Wainwright *et al.*, 1986). Results of my preliminary study suggest that although oosporein lacks antibiotic properties it has, potentially, a novel role in the lifecycle of *B. bassiana* and possibly in those of other oosporein-producing fungi.

Oosporein production in the lifecycle of *B. bassiana* was examined by exposing liquid cultures to light to determine if it can be photo-induced and to confirm if its production is correlated with whole animal virulence. Four *B. bassiana* isolates, exhibiting low to high levels of whole animal virulence were selected to inoculate liquid media and incubated under light (16:8; L:D) at 25°C ± 2°C for 14 days.

1xSDBY liquid medium supported *in vitro* growth of viable *B. bassiana* isolates (Table 2.10). Growth was evident as early as 48 h post inoculation. Figures 2.27 and 2.28 further suggest that isolates GHA and SR-35 had stronger hydrophobic properties than SR-11 and SR-25. This is indicated by the growth of GHA and SR-35 predominately at the air/liquid interface and of SR-11 and SR-25 within the liquid medium.

All 4 *B. bassiana* isolates produced oosporein as early as 48 h post inoculation (SR-35) and as late as 144 h post inoculation (SR-11) based on visual assessment (Table

2.11). Results indicated that all 4 isolates, exhibiting low to high levels of whole animal virulence, produced oosporein thereby disassociating oosporein production from whole animal virulence. Furthermore, isolate SR-11, though exhibiting low whole animal virulence (Table 2.6) produced a large quantity of oosporein (~100% of medium has pigment) while SR-25, having a high degree of whole animal virulence (Table 2.6) produced a large quantity of oosporein. Hence, oosporein production is not necessarily correlated with whole animal virulence. Instead, oosporein may function in the lifecycle of *B. bassiana* as a photo-protectant while the fungus is in the vegetative state. Results further indicated that oosporein can be produced as soon as mycelial growth is visible (SR-35) and as late as 3 days after (GHA). Both isolates SR-11 (low virulence) and SR-25 (high virulence), synthesized oosporein 1 day after growth began. Based on the putative metabolic rate of an isolate or group of isolates and its/their ability to synthesize oosporein in the presence of light, I propose that isolate(s) will produce oosporein at a specific time within early growth for a specific length of time in its vegetative growth phase perhaps to protect itself from potential damage caused by UV-irradiation.

My results also indicated that relative oosporein concentrations within cultures of GHA and SR-3 decreased on day 14. However, SR-11 and SR-25 exhibited no decrease in oosporein production on day 14. Thus some isolates may repress oosporein production during the transition from vegetative to reproductive growth while others may continue to express oosporein production once it has been induced. The fate of oosporein in cultures where colouration decreased in the medium is unknown though it may be degraded and/or re-absorbed by the isolate. It appears that the possible bio-energetic savings of repressing oosporein production versus constitutive production of oosporein (a potentially high energetic cost) does not offer a selective advantage in terms of whole animal virulence as demonstrated with the high virulence of both isolates SR-35 and SR-25.

Vining *et al.* (1962), Brewer *et al.* (1984), Taniguchi *et al.* (1984) and Wainwright *et al.* (1986) suggested oosporein to be effective against Gram-positive but to have little effect on Gram-negative bacteria. Vining *et al.* (1962) concluded that the presence of oosporein in the culture fluid may have antibiotic activity. The lack of clarity of Vining *et al.* (1962) was noted also by Brewer *et al.* (1984). Brewer *et al.* (1984) reported relatively polar compounds, such as oosporein, to have little activity in either bacterial or fungal systems. I believe this conclusion to be premature as Brewer *et al.* (1984) used: (1) solutions for antibacterial tests incorporating ethyl alcohol at unknown stock concentrations which is known to have antiseptic properties; (2) four of eight bacteria were not evaluated and (3) of the four bacteria tested, except for *Micrococcus luteus* (Schroeter) Cohn (minimum inhibitory concentration (MIC), 100 µg/ml) generally very high doses (MIC ≥ 500 µg/ml – 1000 µg/ml) were required. *Micrococcus luteus* is a commonly used bacterium in Gram-positive cell wall research and has high sensitivity to lysozyme; thus it may have increased susceptibility to chemicals used in inhibitory studies (Pickard, personal communication). Based on my observations using standard plate competition assays whereby one organism (e.g. *B. bassiana*) is perpendicularly streaked on an agar plate against competing organisms (e.g. various bacterial or fungal isolates), *B. bassiana* inhibited bacterial isolates in the absence of oosporein (Zurek and Rajput, data not shown).

Taniguchi *et al.* (1984) used culture filtrates to determine antibacterial activity. As with Vining *et al.* (1962), this approach does not provide convincing evidence that oosporein causes antibacterial activity. Wainwright *et al.* (1986) stated that antibacterial activity of oosporein was weak. Erythromycin had a MIC of ≤ 0.5 µg/ml against most of the bacteria tested, whereas oosporein required a MIC of 32 – ≥ 1000 µg/ml. Perhaps the weak antibacterial activity is due to use of questionable purification methods as they indicated that the UV spectrum of an ether extract of acidified (pH 2.0) culture filtrate showed two clearly defined peaks at 288 and 218 nm, which corresponds closely to the spectrum of oosporein. Two peaks may suggest an impurity. Also, as Wainwright *et al.* (1986) do not provide sufficient information on UV spectra (e.g. peak area), UV sensitivity cannot be determined.

Oosporein produced by *B. bassiana* may function in photo-protection. This idea is plausible based on Bandaranayake's (1998) review on UV-absorbing metabolites/compounds as nature's potential sunscreens (i.e. mycosporines). According to Bandaranayake (1998), UV-absorbing mycosporines generally contain one of two cyclic units; an aminocyclohexenone or an aminocyclohexenimine. Fungal metabolites with UV absorption at 310 nm or 320 nm, possess only the aminocyclohexenone ring and are collectively referred to as mycosporines. Mycosporines and mycosporine-like amino acids (MAA) are ubiquitous in terrestrial (including many classes of fungi) and marine organisms and may play an important, though unclarified, role in biological systems. In fungi, mycosporines or their biochemical precursors have always been associated with sporulating mycelia and were considered as biochemical markers for reproductive state (Bandaranayake, 1998). Few instances are known of them having a photo-protective role (Bandaranayake, 1998). The synthesis of these metabolites is regulated by light intensity and spectral composition (Bandaranayake, 1998). Oosporein may have some relation to mycosporines, which confer either a photo-protective role to vegetative mycelia or facilitate other aspects of fungal growth besides induction of sporulation as *B. bassiana* can sporulate under dark conditions.

More isolates of *B. bassiana* need to be assessed to confirm these results. Oosporein-infused culture filtrates should be sterilized by syringe filtration and subjected to spectrophotometry analyses to quantify oosporein production and to determine UV absorption maxima. How does the possibility of photo-protection translate *in vivo*? Future studies are required to confirm the role(s) of oosporein in the lifecycle of *B. bassiana* as a putative mycelial photo-protectant and if, indeed, oosporein is related to naturally occurring UV-absorbing compounds known as mycosporines.

2.5. Summary / Conclusions

Beauveria bassiana isolated from numerous arthropod species was assessed for control potential in an integrated pest management program for western flower thrips, *F. occidentalis*. The conventional approach to identify highly virulent isolates by whole animal bioassays is time-consuming and labour intensive, which decreases isolate screening efficiency. My approach integrated a standard testing procedure using indirect exposure of target insect species and selected *in vitro* morphological growth characteristics. Isolates of *B. bassiana* exhibiting characteristics of good/excellent harvest quality, thin or very few (i.e. powder-like conidia) mycelial fragments and a none/moderate degree of submerged mycelia will have a high probability of having high *in vivo* virulence. Isolates of *B. bassiana* exhibiting characteristics of poor/moderate harvest quality, thick mycelial fragments and a high degree of submerged mycelia will have a high probability of having low *in vivo* virulence. However, it is important that isolates exhibit generally consistent growth morphologies for accurate *in vivo* virulence predictions to be made. These characteristics clearly exhibited a pattern relating to whole animal virulence based on median lethal times and provide a valuable tool for increasing screening efficiency of potentially virulent isolates for insect pest management. Oosporein production in the lifecycle of *B. bassiana* was also examined by photo-exposure to determine if it can be photo-induced and to confirm if its production is correlated with whole animal virulence. Results indicated that all 4 isolates tested, exhibiting low to high levels of whole animal virulence, produced oosporein thereby disassociating oosporein production from whole animal virulence. Instead, oosporein may function in the lifecycle of *B. bassiana* as a photo-protectant while the fungus is in the vegetative state.

2.6. Tables and figures

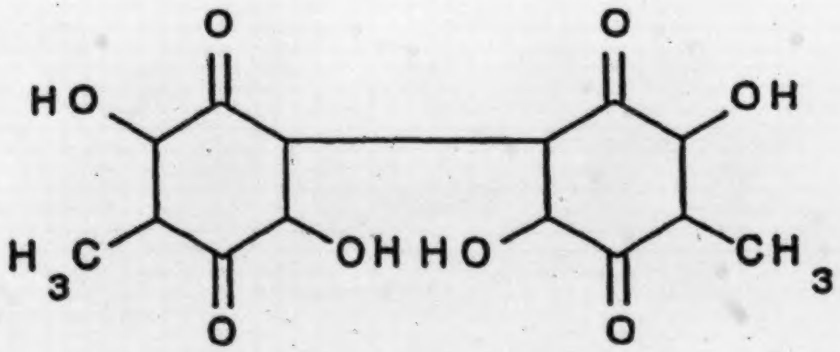

Figure 2.1. Chemical structure of oosporein (Eyal *et al*., 1994).

Figure 2.2. *Beauveria bassiana* isolate SR-51 cultured on 1xSDAY (1% YE) solid medium at 23°C ± 2°C for 14 days. (A) Culture incubated in the presence of light. (B) Culture incubated in the dark. Natural medium colour is amber as seen in (B) however, red pigment indicates oosporein infusion as seen in (A).

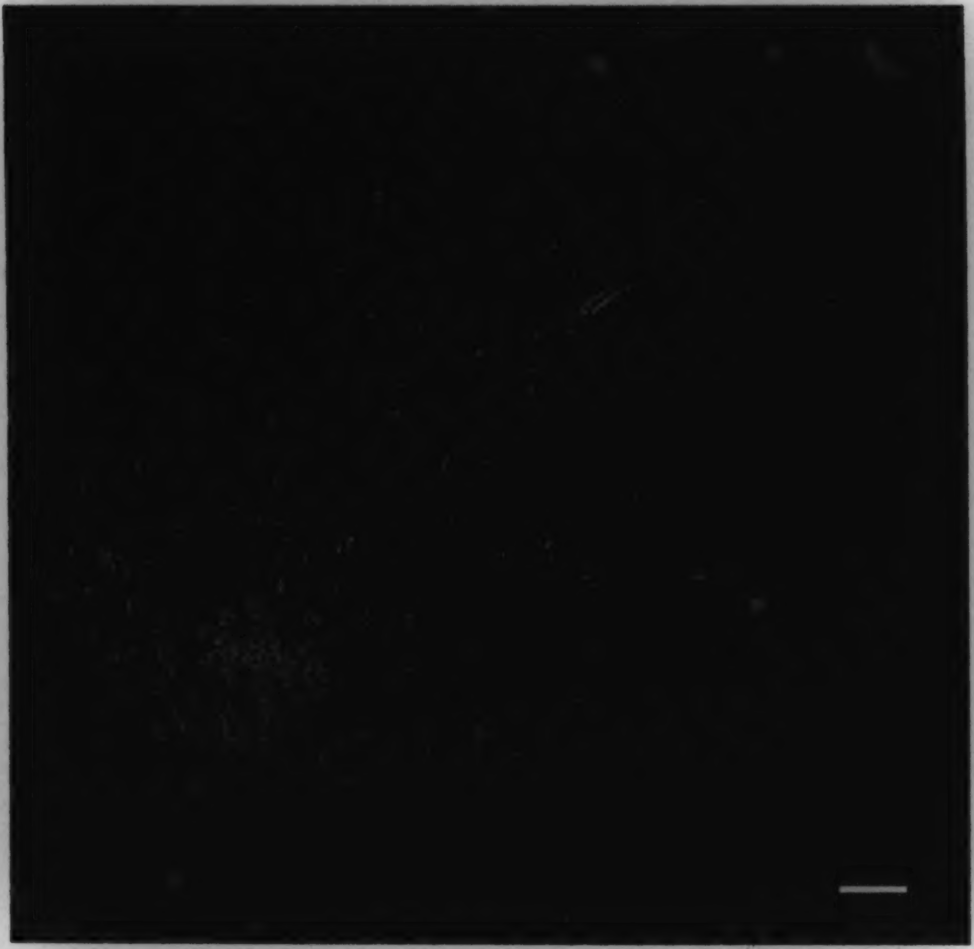

Figure 2.3. Adult female western flower thrips (*F. occidentalis*) feeding on bush bean leaf (*Phaseolus vulgaris*) and non-viable apple pollen (yellow granules) as a protein supplement. Image taken with a Nikon Coolpix® 775 digital camera using a Leica Wild® M3C stereomicroscope. Dorsal view. Scale bar = 1 mm.

Table 2.1. Description of *Beauveria bassiana* isolates selected for this study.

Isolate identity	Host (order)	Geographic origin	Source
SR-1	Lepidoptera	Canada	B.A. Keddie[b]
SR-2	Lepidoptera	Canada	S. Rajput[b] /B.A. Keddie[b]
SR-3	Lepidoptera	Canada	B.A. Keddie[b]
SR-7	Hemiptera	Canada	M. Greif[b]
SR-9	Hymenoptera	Canada	M. Greif[b]
SR-11	Hymenoptera	Canada	M. Greif[b]
SR-12	Coleoptera	Canada	M. Greif[b]
SR-13	Coleoptera	Canada	M. Greif[b]
SR-15	Araneae	Canada	M. Greif[b]
SR-16	Diptera	Canada	M. Greif[b]
SR-19	Araneae	Canada	M. Greif[b]
SR-21	Araneae	Canada	M. Greif[b]
SR-22	Araneae	Canada	M. Greif[b]
SR-23	Diptera	Canada	M. Greif[b]
SR-24	Araneae	Canada	M. Greif[b]
SR-25	Acari	Canada	M. Greif[b]
SR-35	Hemiptera	Canada	K. Fry[c]
SR-36	Hemiptera	Canada	K. Fry[c]
SR-41	Coleoptera	Canada	M. Greif[b]
GHA	Coleoptera[a]	USA	Emerald BioAgriculture[d]

[a] According to Becker (2000). Currently, there is much uncertainty as to the original host for isolate GHA (Humber, personal communication).

[b] Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada.

[c] School of Horticulture, Olds College, Olds, Alberta, Canada.

[d] Formerly Mycotech.

FUNGAL INCUBATION IN PROGRESS
PLEASE DO NOT DISTURB
FUNGAL INCUBATION IN PROGRESS
PLEASE DO NOT DISTURB

Figure 2.4. Dark incubation chambers. Interior chambers were lined with thick black construction paper and any potential light leaks were sealed with masking tape. No light penetration (zero foot-candles) was confirmed using a hand-held light meter. This and other images taken with Nikon Coolpix® 775 digital camera.

Figure 2.5. Passive drying chambers. This system allowed for simultaneous drying of isolates within individual compartments.

Figure 2.6. *Beauveria bassiana* isolate SR-15 harvested into a sterile 60 x 15 mm petri dish, sealed with parafilm and wrapped up in a thick plastic poly bag secured with 2 rubber bands; ready for 4°C storage under desiccation.

Figure 2.7. *Beauveria bassiana* isolate SR-3 harvested into a sterile 60 x 15 mm petri dish exhibiting the following morphological characteristics: excellent harvest quality and thin mycelial fragments ("Growth" category: 32).

Figure 2.8. *Beauveria bassiana* isolate SR-15 harvested into a sterile 60 x 15 mm petri dish exhibiting the following morphological characteristics: poor harvest quality and thick mycelial fragments ("Growth" category: 9).

Figure 2.9. *Beauveria bassiana* isolate SR-35 harvested into a sterile 60 x 15 mm petri dish exhibiting the following morphological characteristics: excellent harvest quality and powder-like conidia ("Growth" category: 29).

Figure 2.10. *Beauveria bassiana* conidia exposed to a working stock of 1.2% (volume/volume) propidium iodide solution to determine isolate viability. Preparation was further exposed to ~510 nanometers (green-light) via epifluorescence causing conidia to fluoresce red. Viable conidia appear as red halos as the intact cell membranes are excluding propidium iodide. Non-viable conidia appear as solid red spheres (arrow) as the compromised cell membrane has permitted the vital dye to enter and intercalate within the DNA as clearly seen. Scale bar = 2 µm. Fuji Elite Chrome colour film (ISO 400).

Table 2.2. *Beauveria bassiana* isolates selected for this study based on western flower thrips whole animal virulence pooled screening trials.

Trial	Isolate	LT_{50} (days)
1[a]	SR-1	4.66
	SR-2	4.25
	SR-3	4.50
	SR-7	5.56
	SR-9	5.28
	SR-11	5.32
	SR-12	5.31
	SR-13	4.86
	SR-15	4.78
	SR-16	5.39
	SR-19	4.39
	SR-21	4.56
	SR-22	3.47
	SR-23	4.43
	SR-24	4.57
	SR-25	3.88
	SR-35	4.95
	SR-36	4.35
	SR-41	4.52
	GHA	6.17[b]

[a] Pooled screening trials (n = 7).

[b] Pooled isolate GHA from all screening trials (n = 7). Standard error = ±0.33.

Table 2.3. *Beauveria bassiana* isolates selected for this study based on western flower thrips whole animal performance in evaluation trial 1.

Evaluation trial	Isolate	LT_{50} (days)	$\pm S.E._{LT50}$ (days)
1[a]	SR-3	4.73	0.11
	SR-15	7.71	1.33
	SR-35	4.80	0.20
	SR-37	5.54	0.24
	GHA	6.03	0.40

[a] Pooled evaluation trials (n = 3). Mean LT_{50} value reported.

Table 2.4. *Beauveria bassiana* isolates selected for this study based on western flower thrips whole animal performance in evaluation trial 2.

Evaluation trial	Isolate	LT_{50} (days)	$\pm S.E._{LT50}$ (days)
2[a]	SR-7	4.91	0.33
	SR-11	N/D[b]	N/D[b]
	SR-25	4.94	0.34
	GHA	5.88	0.28

[a] Pooled evaluation trials (n = 3). Mean LT_{50} value reported.
[b] Non-discernable response. Actual response value = 2.11E+13.

Table 2.5. Statistical matrix used to code category "growth" as it consisted of three characteristics for the purposes of increased accuracy and incorporating any minor variation with isolates of *Beauveria bassiana*.

Growth	Harvest quality	Mycelial fragments	Submerged mycelia
1	1	1	1
2	1	1	2
3	1	1	3
4	1	2	1
5	1	2	2
6*	1	2	3
7	1	3	1
8*	1	3	2
9*	1	3	3
10	2	1	1
11	2	1	2
12	2	1	3
13	2	2	1
14	2	2	2
15	2	2	3
16	2	3	1
17	2	3	2
18	2	3	3
19	3	1	1
20	3	1	2
21	3	1	3
22	3	2	1
23*	3	2	2
24*	3	2	3
25	3	3	1
26*	3	3	2
27	3	3	3
28*	4	1	1
29*	4	1	2
30	4	1	3
31	4	2	1
32*	4	2	2
33	4	2	3
34	4	3	1
35	4	3	2
36	4	3	3

***Indicates a categorical code used for an isolate of *Beauveria bassiana* in either screening or evaluation trials. In some cases, categorical code was used more than once.**

Table 2.6. *Beauveria bassiana* isolates selected based on whole animal virulence to determine any relationship to oosporein production.

Isolate	LC_{50} (conidia/ml)	± $S.E._{LC50}$ (conidia/ml)	LT_{50} (days)	± $S.E._{LT50}$ (days)
SR-11[a]	8.28E+09	8.07E+09	N/D[c]	N/D[c]
SR-25[a]	3.52E+07	1.12E+07	4.94	0.34
SR-35[a]	2.67E+07	4.30E+06	4.80	0.20
GHA[b]	2.48E+09	1.31E+09	5.95	0.22

[a] Pooled trials (n = 3). Mean LC_{50} and LT_{50} values reported.
[b] Pooled trials (n = 6). Mean LC_{50} and LT_{50} values reported.
[c] Non-discernable response. Actual response value = 2.11E+13.

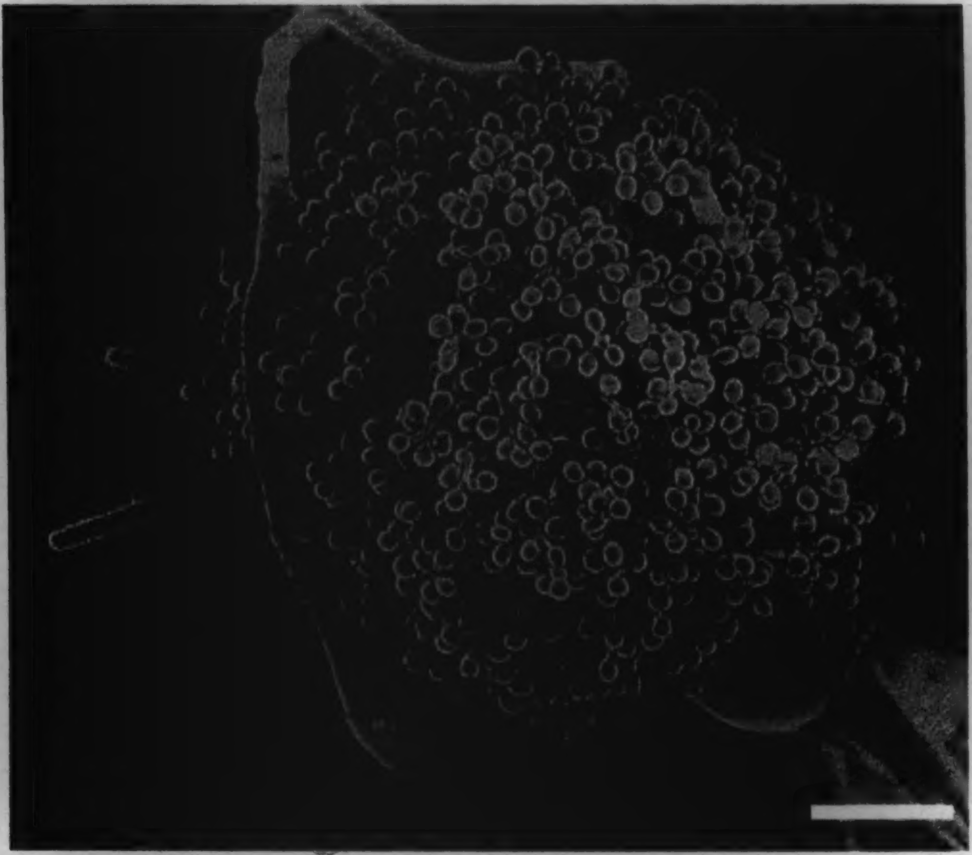

Figure 2.11. Scanning electron micrograph of *Beauveria bassiana* conidiophore and conidia (arrow). Scale bar = 10 µm. Image courtesy of Dr. B.A. Keddie.

Figure 2.12. *Beauveria bassiana* isolate SR-11 cultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days and passively dried for 2 days; ready for harvesting.

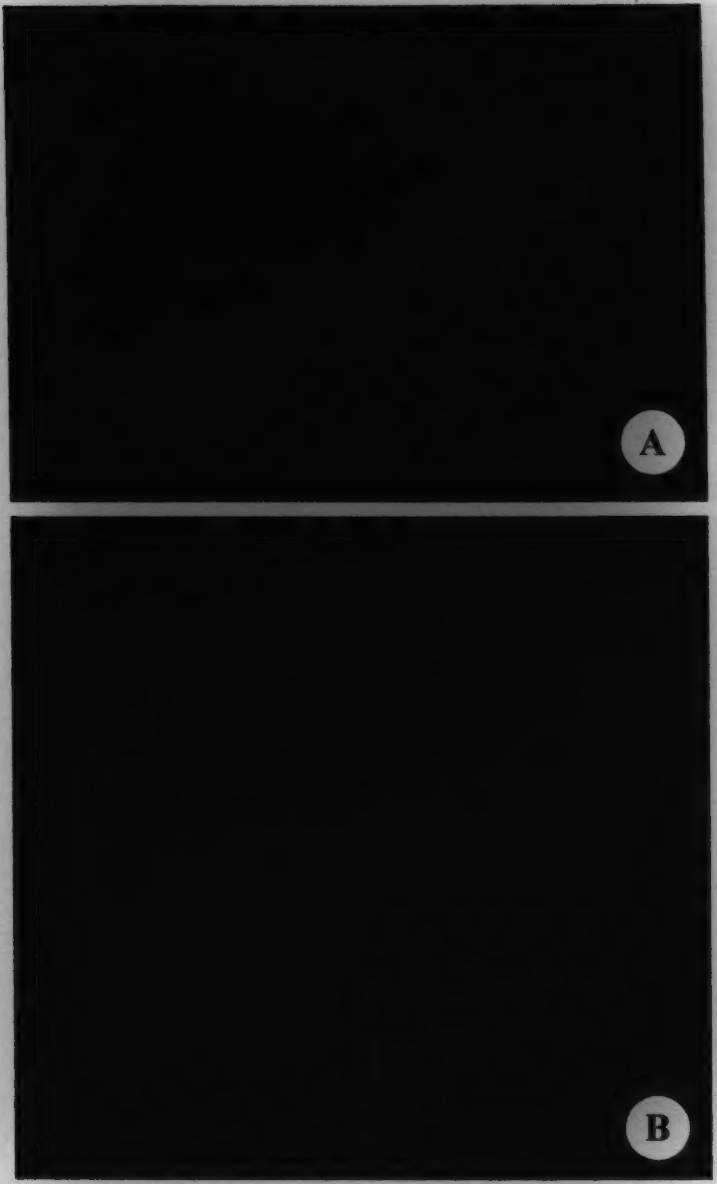

Figure 2.13. *Beauveria bassiana* isolate SR-11 cultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days and passively dried for 2 days before being harvested exhibiting the following morphological characteristics: poor harvest quality, thick mycelial fragments and moderate degree of submerged mycelia ("Growth" category: 8). (A) and (B) are taken at different angles of view.

Figure 2.14. *Beauveria bassiana* isolate SR-11 harvested into a sterile 60 x 15 mm petri dish exhibiting the following morphological characteristics poor harvest quality and thick mycelial fragments ("Growth" category: 8).

Figure 2.15. *Beauveria bassiana* isolate SR-15 cultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days and passively dried for 2 days before being harvested exhibiting the following morphological characteristics: poor harvest quality and deeply submerged mycelia ("Growth" category: 9).

Figure 2.16. *Beauveria bassiana* isolate SR-15 harvested into a sterile 60 x 15 mm petri dish exhibiting the following morphological characteristics: poor harvest quality and thick mycelial fragments ("Growth" category: 9).

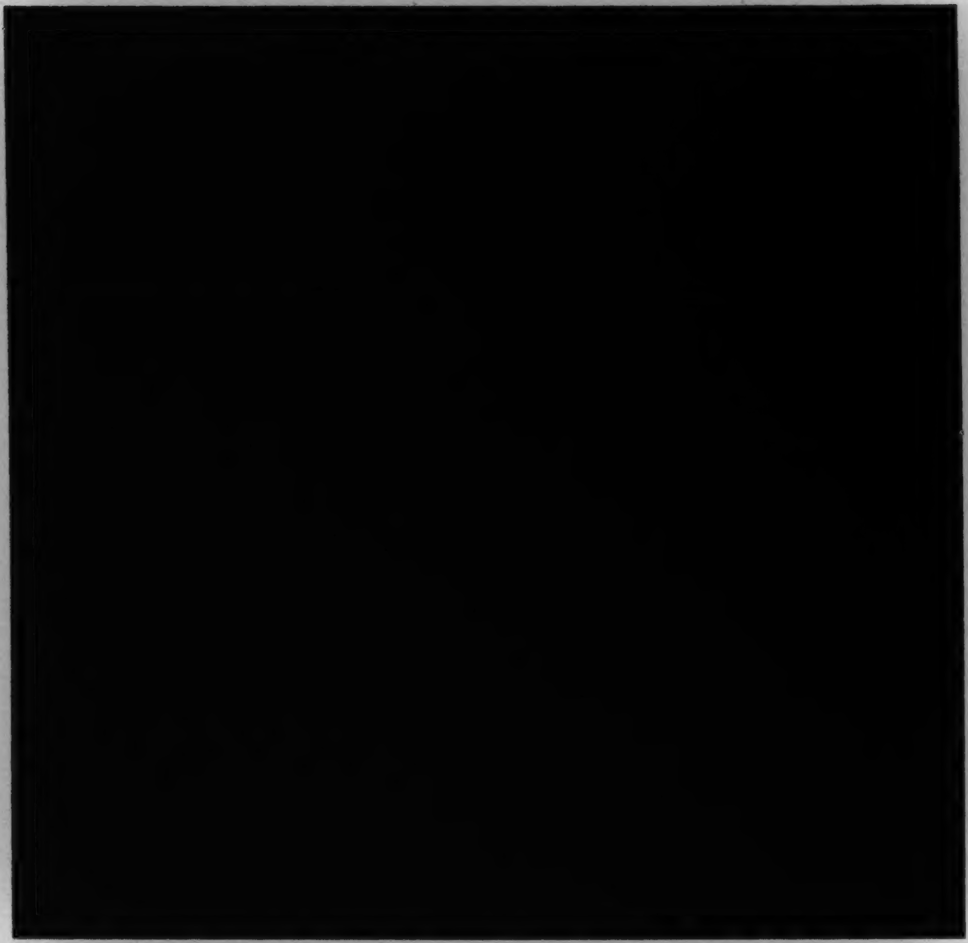

Figure 2.17. *Beauveria bassiana* isolate SR-25 cultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days and passively dried for 2 days; ready for harvesting.

Figure 2.18. *Beauveria bassiana* isolate SR-25 cultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days and passively dried for 2 days before being harvested exhibiting the following morphological characteristic: moderately submerged mycelia ("Growth" category: 23).

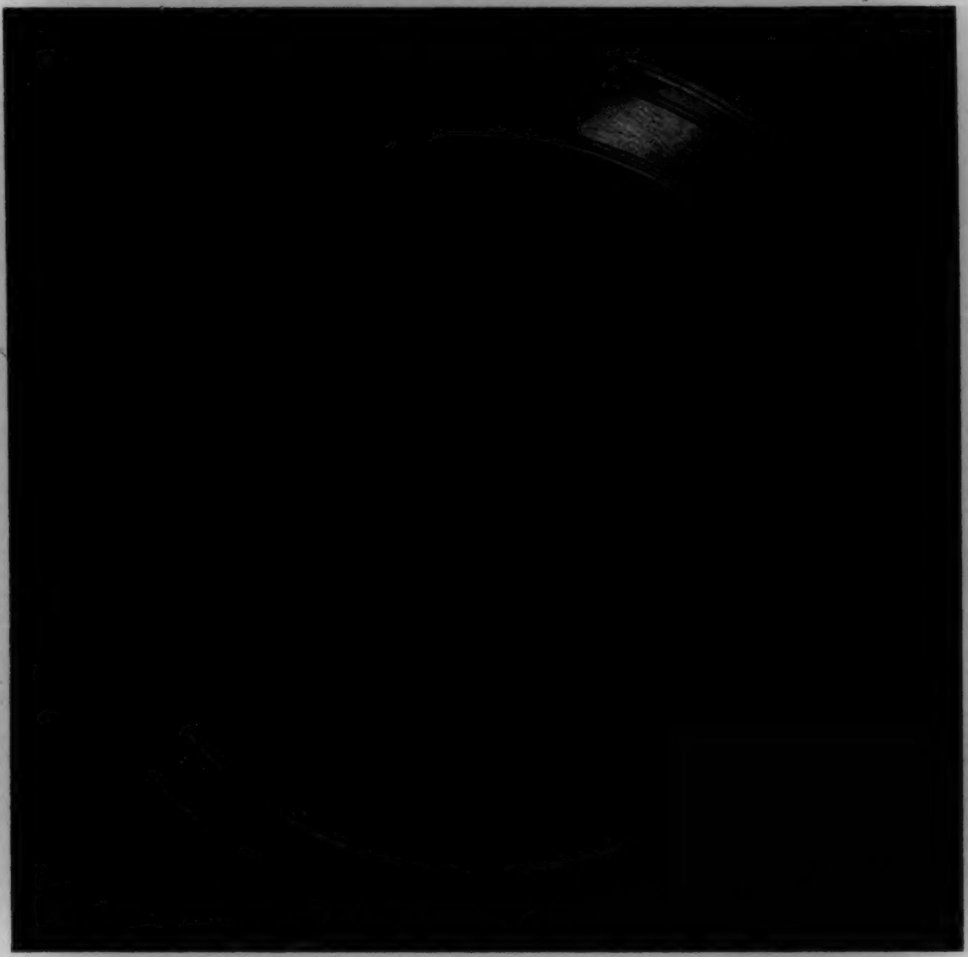

Figure 2.19. *Beauveria bassiana* isolate SR-25 harvested into a sterile 60 x 15 mm petri dish exhibiting the following morphological characteristics: good harvest quality and thin mycelial fragments ("Growth" category: 23).

Figure 2.20. *Beauveria bassiana* isolate SR-3 cultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days and passively dried for 2 days before being harvested exhibiting the following morphological characteristic: moderately submerged mycelia ("Growth" category: 32).

Figure 2.21. *Beauveria bassiana* isolate SR-3 harvested into a sterile 60 x 15 mm petri dish exhibiting the following morphological characteristics: excellent harvest quality, thin mycelial fragments ("Growth" category: 32).

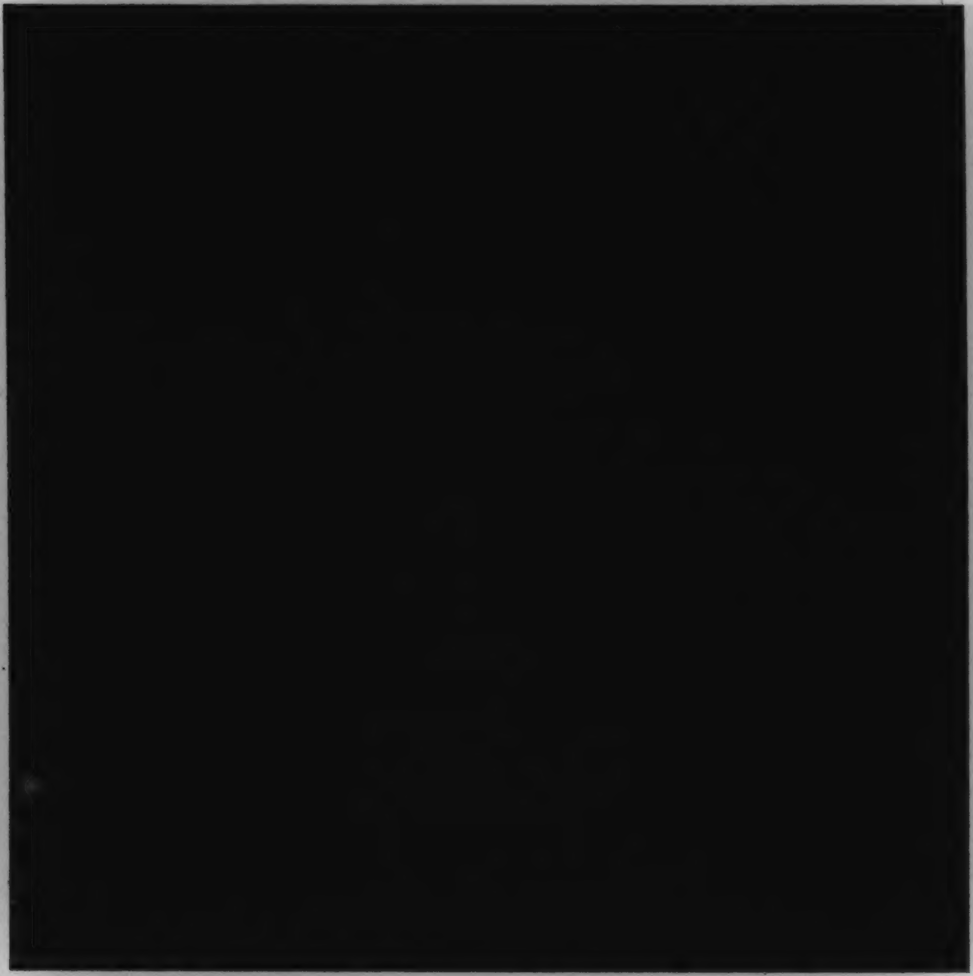

Figure 2.22. *Beauveria bassiana* isolate SR-35 cultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days and passively dried for 2 days; ready for harvesting.

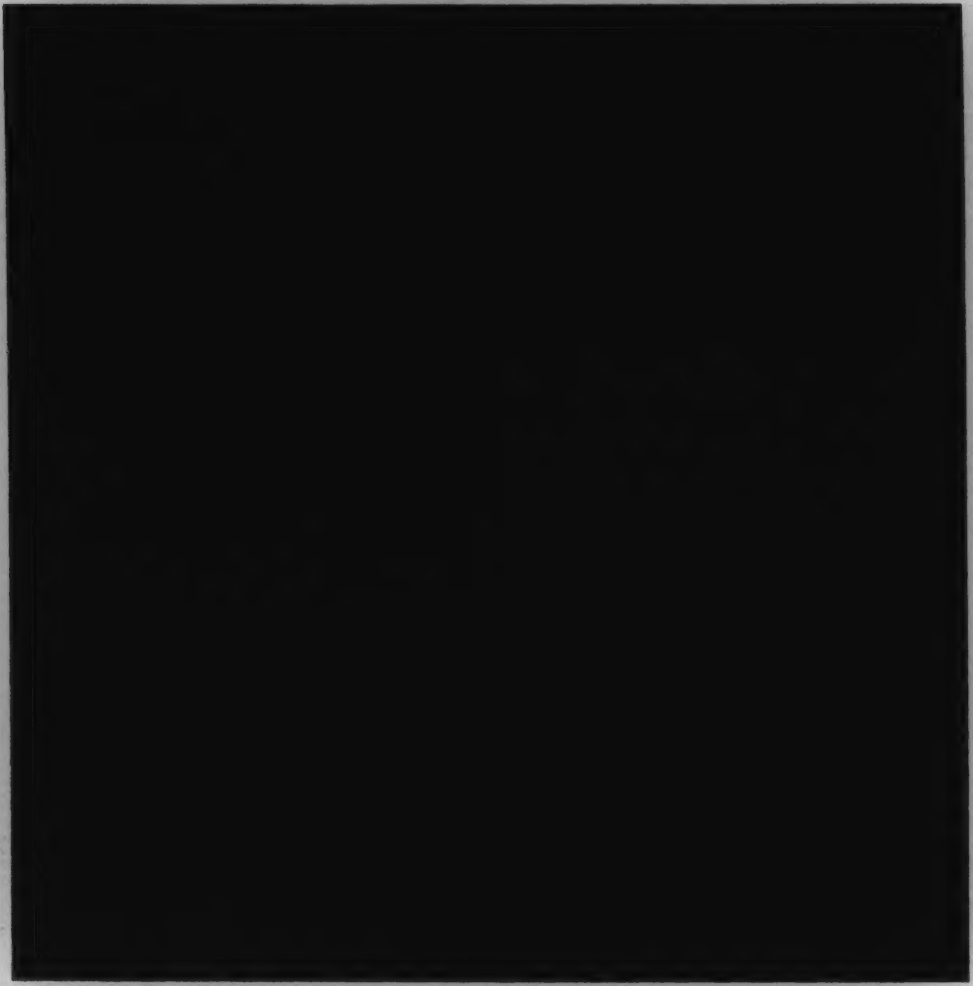

Figure 2.23. *Beauveria bassiana* isolate SR-35 cultured on 0.5xSDAY in the dark at 23°C ± 2°C for 14 days and passively dried for 2 days before being harvested exhibiting the following morphological characteristics: excellent harvest quality, powder-like conidia and moderately submerged mycelia ("Growth" category: 29).

Figure 2.24. *Beauveria bassiana* isolate SR-35 harvested into a sterile 60 x 15 mm petri dish exhibiting the following morphological characteristics: excellent harvest quality and powder-like conidia ("Growth" category: 29).

Table 2.7. Type III sum of squares (SS) analyses of *in vitro* morphological growth characteristics of *Beauveria bassiana* isolates within pooled trials using LT_{50} data from western flower thrips bioassays to determine if any characteristics can be used for increasing isolate screening efficiency.

Trial	Category	DF	F-Value	P-Value
Screen[a]	Growth[c]	7	3.47	0.0324
	Aerial	1	0.01	0.9244
	Yellow	1	1.65	0.2160
	Red	1	0.25	0.6236
Evaluation[b]	Growth	3	5.18	0.0179
	Aerial	1	0.93	0.3531
	Yellow	1	0.93	0.3531
	Red	1	0.25	0.6246

[a] Pooled screening trails (n = 7).

[b] Pooled evaluation trails (n = 3).

[c] Consists of 3 combined characteristics: harvest quality, mycelial fragments and degree of mycelial submergence.

Table 2.8. Viability of *Beauveria bassiana* isolates used in screening trials.

Trial	Isolate	Viability (%)
1[a]	SR-1	99.5
	SR-2	96.9
	SR-3	99.7
	SR-7	45.3
	SR-9	99.7
	SR-11	99.2
	SR-12	97.0
	SR-13	85.0
	SR-15	96.4
	SR-16	99.5
	SR-19	98.4
	SR-21	97.8
	SR-22	99.3
	SR-23	99.8
	SR-24	98.9
	SR-25	96.5
	SR-35	99.3
	SR-36	99.1
	SR-41	97.4
	GHA	91.7[b]

[a] Pooled screening trails (n = 7).

[b] Pooled isolate GHA from all screening trials (n = 7). Standard deviation = ±5.4%.

Table 2.9. Viability of *Beauveria bassiana* isolates used in evaluation trials.

Trial	Isolate	Viability (%)
1a[a]	SR-3	99.9
	SR-15	99.0
	SR-25	88.9
	SR-35	99.3
	SR-37	99.3
	GHA	96.7[d]
1b[b]	SR-3	99.8
	SR-15	99.0
	SR-25	98.2
	SR-35	100.0
	SR-37	99.5
	GHA	94.4[e]
1c[c]	SR-3	98.8
	SR-15	97.5
	SR-25	93.6
	SR-35	99.0
	SR-37	94.7
	GHA	95.1[f]

[a] Pooled evaluation trails 1a and 2a.
[b] Pooled evaluation trails 1b and 2b.
[c] Pooled evaluation trails 1c and 2c.
[d] Standard deviation = ±1.5% (n = 2).
[e] Standard deviation = ±4.8% (n = 2).
[f] Standard deviation = ±2.6% (n = 2).

Table 2.10. *In vitro* growth of *Beauveria bassiana* isolates varying in whole animal virulence against *Frankliniella occidentalis* by exposure to light at 25°C ± 2°C, 16:8 h (light:dark) for 14 days to determine if conditions were suitable for fungal growth.

Flask #	Isolate	0 h (0 d)	24 h (1 d)	48 h (2 d)	72 h (3 d)	96 h (4 d)	120 h (5 d)	144 h (6 d)	168 h (7 d)	336 h (14 d)
1	Negative control[a]	-[b]	-	-	-	-	-	-	-	-
2	GHA	-	-	+	+	+	++	++	++	++
3	SR-11	-	-	+[c]	+	+	+	+	+	+
4	SR-25	-	-	+[c]	+	+	+	+	+	+
5	SR-35	-	-	+	+	+	++	++	++	++

[a] Non-inoculated flask.

[b] Symbol: -, negative (no visible) growth; +, mycelia evident; ++, confluent mycelial mat at the air/liquid interface.

[c] Very sparse mycelial growth relative to isolates GHA and SR-35.

Table 2.11. *In vitro* induction of oosporein in *Beauveria bassiana* isolates by exposure to light at 25°C ± 2°C, 16:8 h (light:dark) for 14 days.

Flask #	Isolate	0 h (0 d)	24 h (1 d)	48 h (2 d)	72 h (3 d)	96 h (4 d)	120 h (5 d)	144 h (6 d)	168 h (7 d)	336 h (14 d)
1	Negative control[a]	-[b]	-	-	-	-	-	-	-	-
2	GHA	-	-	-	-	-	+	+	+	+/-
3	SR-11	-	-	-	-	-	-	+	++	+++
4	SR-25	-	-	-	+	++	+++	+++	+++	+++
5	SR-35	-	-	+	+	+	+	+	+	+/-

[a] Non-inoculated flask.

[b] Symbol: -, 0% of medium has pigment (i.e. negative pigment production); +, <50% of medium has pigment; ++, ~50% of medium has pigment; +++, >50% (~100%) medium has pigment; +/-, an apparent decrease in pigment concentration is observed.

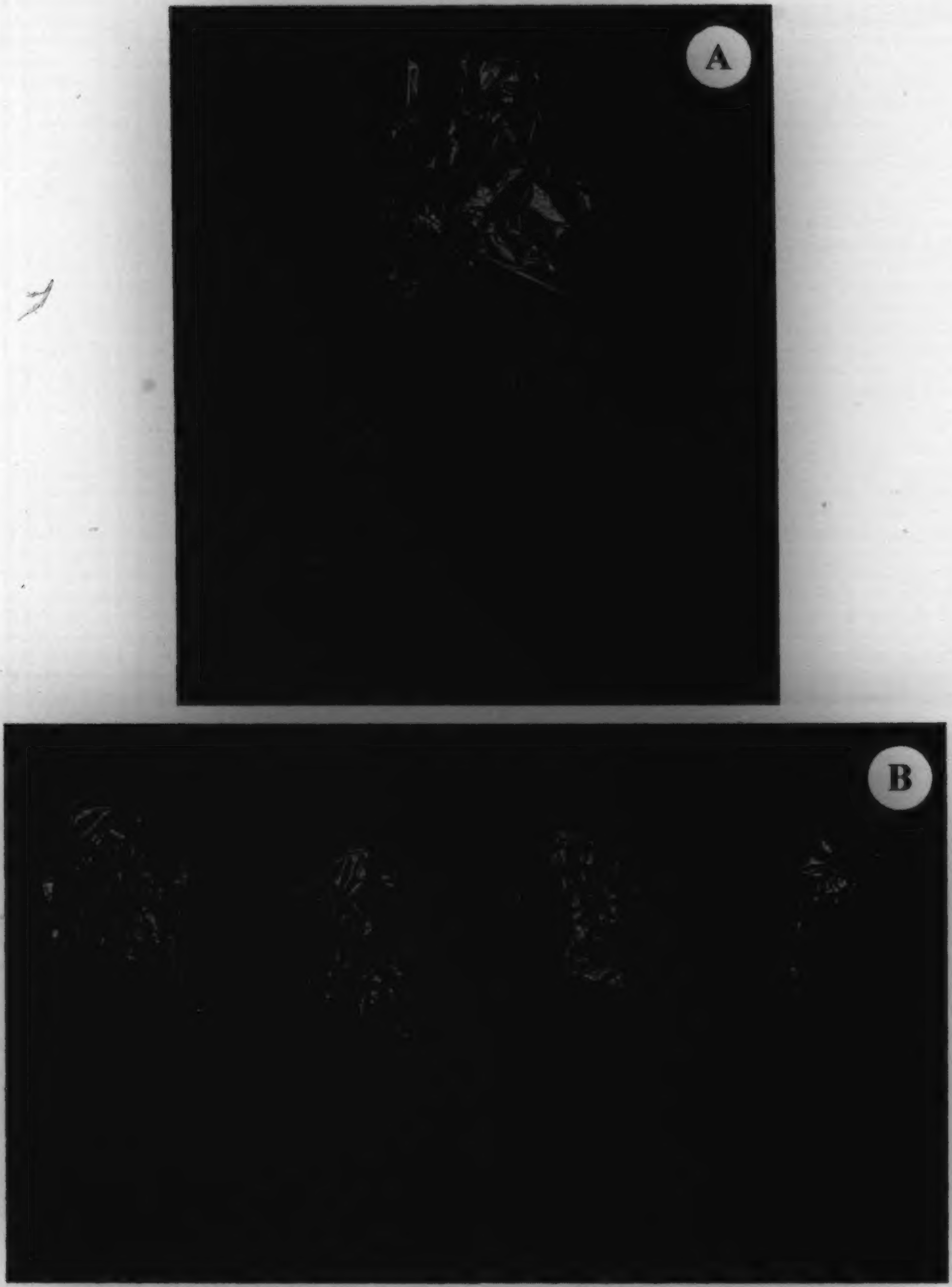

Figure 2.25. Liquid cultures of 1xSDAY medium inoculated with isolates of *Beauveria bassiana* exposed to cool-white light to determine if oosporein can be induced. Isolates are incubated at 25°C ± 2°C, 16:8 h (L:D). (A) indicates a non-inoculated negative control to show general medium colour. (B) indicates the isolates used within this study (from left to right): GHA (TP), SR-11, SR-35 and SR-25. Images taken at 24 hours (day 1).

Figure 2.26. Liquid cultures of 1xSDAY medium inoculated with isolates of *Beauveria bassiana* exposed to cool-white light to determine if oosporein can be induced. Isolates are incubated at 25°C ± 2°C, 16:8 h (L:D). (A) indicates isolates (from left to right): GHA (TP) and SR-11. (B) indicates isolates (from left to right): SR-35 and SR-25. Images at 48 hours (day 2).

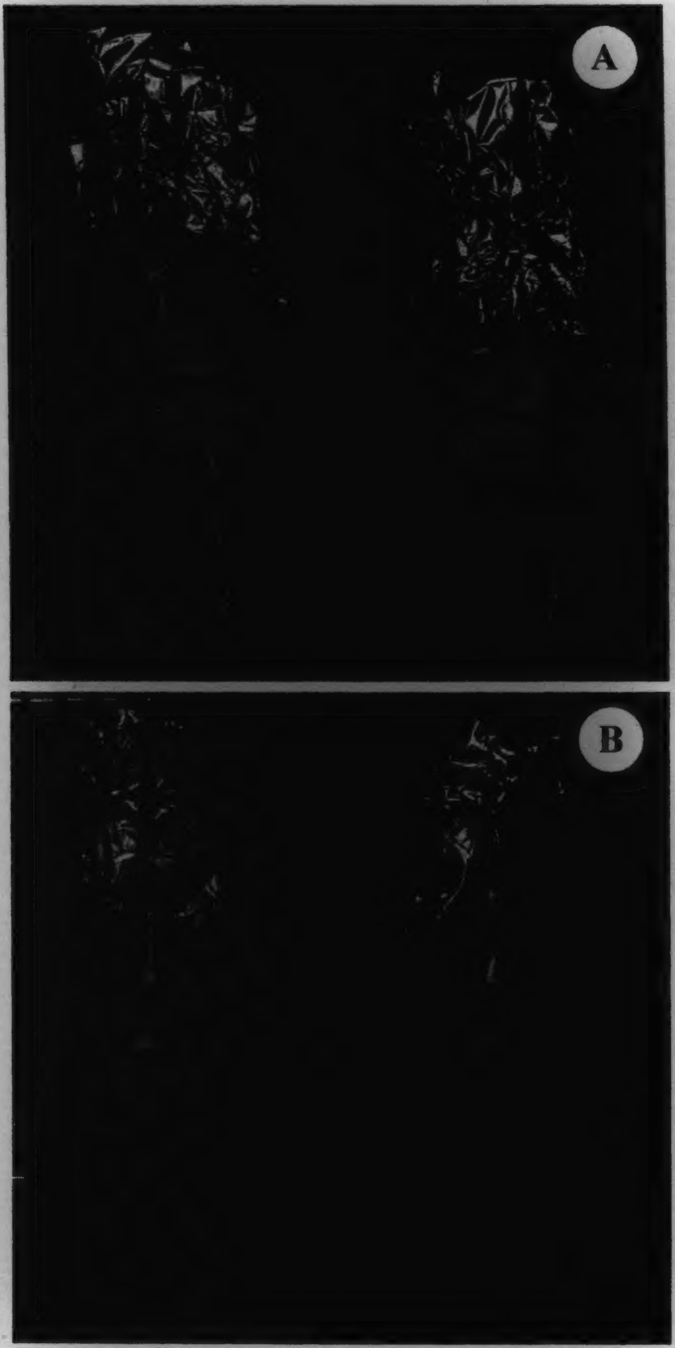

Figure 2.27. Liquid cultures of 1xSDAY medium inoculated with isolates of *Beauveria bassiana* exposed to cool-white light to determine if oosporein can be induced. Isolates are incubated at 25°C ± 2°C, 16:8 h (L:D). (A) indicates isolates (from left to right): GHA (TP) and SR-11. (B) indicates isolates (from left to right): SR-35 and SR-25. Images at 72 hours (day 3).

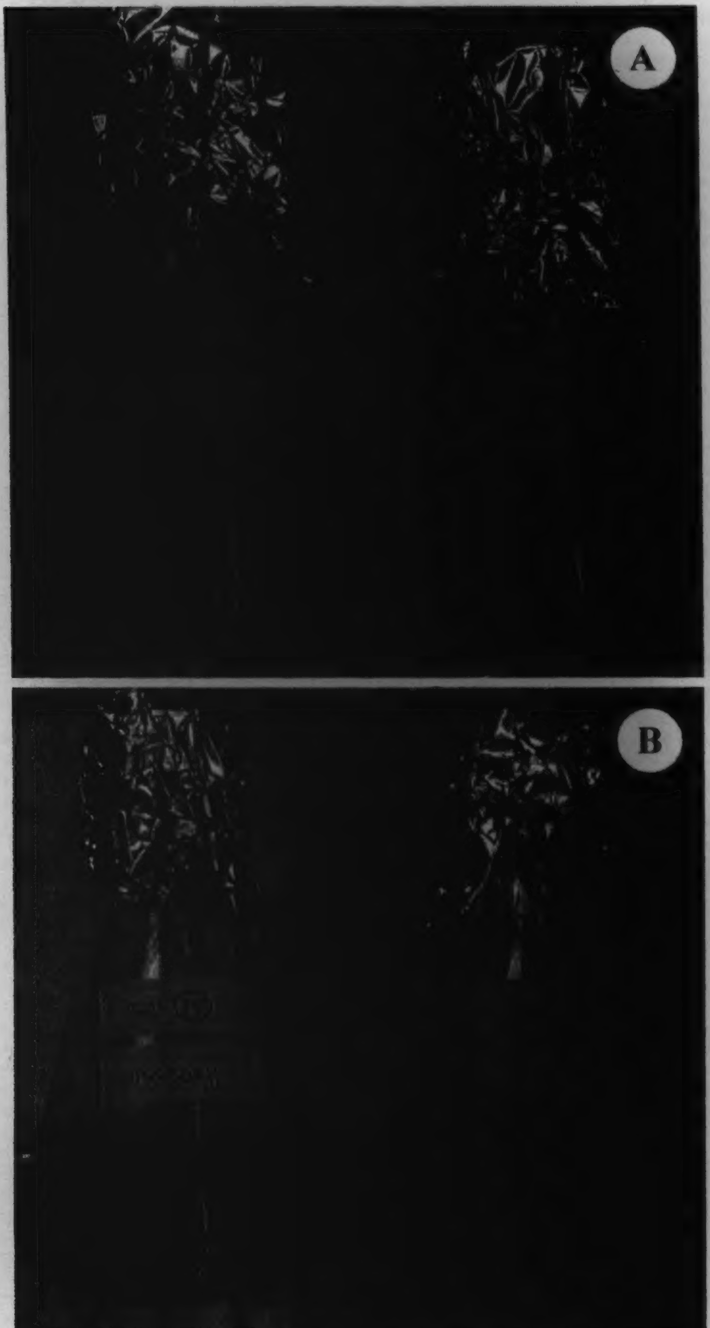

Figure 2.28. Liquid cultures of 1xSDAY medium inoculated with isolates of *Beauveria bassiana* exposed to cool-white light to determine if oosporein can be induced. Isolates are incubated at 25°C ± 2°C, 16:8 h (L:D). (A) indicates isolates (from left to right): GHA (TP) and SR-11. (B) indicates isolates (from left to right): SR-35 and SR-25. Images taken at 120 hours (day 5).

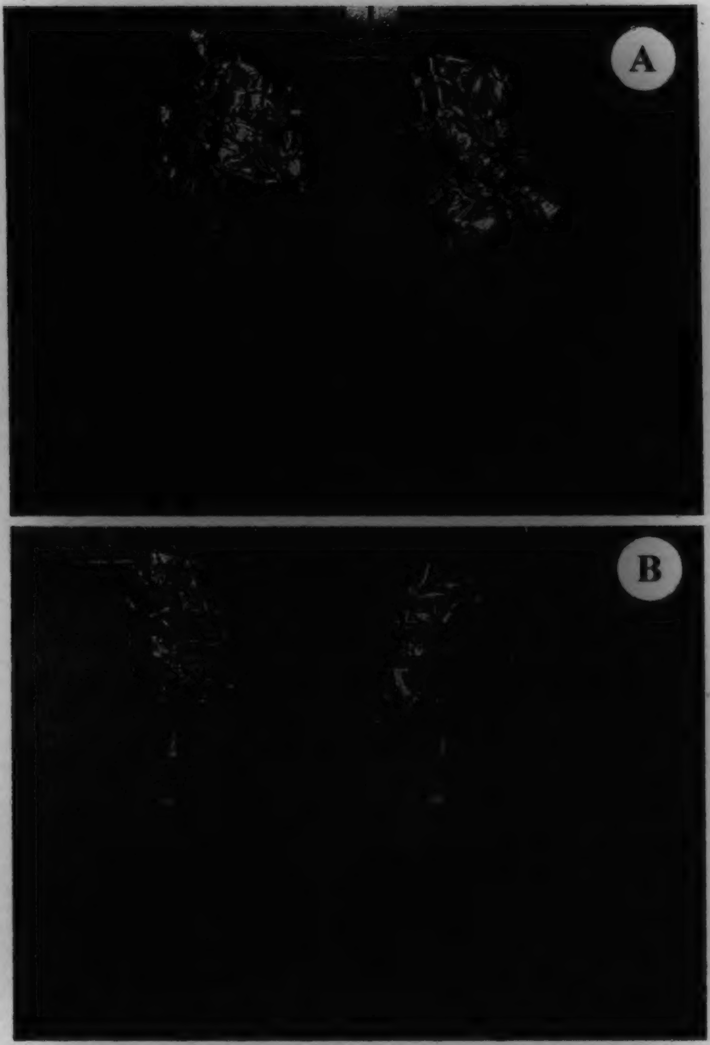

Figure 2.29. Liquid cultures of 1xSDAY medium inoculated with isolates of *Beauveria bassiana* exposed to cool-white light to determine if oosporein can be induced. Isolates are incubated at 25°C ± 2°C, 16:8 h (L:D). (A) indicates isolates (from left to right): GHA (TP) and SR-11. (B) indicates isolates (from left to right): SR-35 and SR-25. Images taken at 336 hours (day 14).

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Chapter 3. Evaluation of beauvericin as a marker for *Beauveria bassiana* virulence and its implication for greenhouse pest management.

3.1. Introduction

Since Agostino Bassi first described the entomopathogenic fungus in 1807 (Ainsworth, 1956), *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) has been studied extensively for its unique properties and its potential for biological control. Until recently, *B. bassiana* was placed in the Phylum Deuteromycota (Fungi Imperfecti) containing many other entomopathogenic and phytopathogenic fungi such as *Paecilomyces* species and *Fusarium* species, respectively (Ainsworth *et al*., 1983; Alexopoulos *et al*., 1996).

Insect pests, such as western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae), are of great concern to the greenhouse ornamental and vegetable industries due to their potential for causing extensive damage and revenue losses (Lewis, 1997). At present, biological control agents, such as entomopathogenic fungi, are used as an alternative to synthetic chemicals for greenhouse pest control. Due to the broad host range of many entomopathogenic fungi and some other biological control agents, screening efforts are required to determine which isolates are best for targeting a specific pest insect and have the highest levels of virulence.

To evaluate entomopathogenic fungi for increased efficacy or high levels of virulence, conventionally, *in vivo* studies using whole animals are conducted. Although whole insect bioassays provide useful information on toxicity towards a target organism, such tests are laborious and time consuming (Fornelli *et al*., 2004). Another approach is to screen fungal isolates based on their metabolites (i.e. mycotoxins). Mycotoxins should be considered due to cereal crop contamination (Logrieco *et al.*, 2002a) and/or their pathological function(s) for some entomopathogens, such as *B. bassiana* (Fuguet and Vey, 2004).

In this study, *B. bassiana* isolates previously evaluated for virulence against western flower thrips (Litwinowich, 2005), are further evaluated for beauvericin production and to determine if beauvericin can be used as an indicator of whole animal virulence.

As discussed by Logrieco *et al.* (2002a), beauvericin initially was reported to be synthesized by entomopathogenic fungi such as *B. bassiana* (Hamill *et al.*, 1969) and *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Bernardini *et al.*, 1975). Subsequently, beauvericin has been detected in numerous entomopathogenic and phytopathogenic isolates of *Fusarium* species (Gupta *et al.*, 1991; Logrieco *et al.*, 1993a; Logrieco *et al.*, 1993b; Moretti *et al.*, 1995; Logrieco *et al.*, 1998; Logrieco *et al.*, 2002b). Structurally, beauvericin ($C_{45}H_{57}N_3O_9$) is a lipophilic cyclic hexadepsipeptide consisting of repeating D-∝-hydrossyisovaleryl-L-*N*-methyl-phenylalanine units (Figure 3.1) and has a molecular weight of 784 (Hamill *et al.*, 1969; Thakur and Smith, 1997). Beauvericin belongs to the family of enniatins, which exert antibiotic activities on Gram-positive bacteria and mycobacteria (Logrieco *et al.*, 2002a). Antimicrobial properties associated with beauvericin are due to its catalyzing the translocation of cations (cationophoric) across the lipid bilayer in the direction of the electrochemical gradient (Thaker and Smith, 1997). Hence, it has been demonstrated that beauvericin has ionophoric properties by forming complexes with monovalent and divalent cations that activates their transport into mammalian mitochondria and through biological and artificial membranes (Logrieco *et al*., 2002a). Furthermore, Ojcius *et al*. (1991) report that beauvericin induces programmed cell death (apoptosis) via calcium-activated endonucleases causing cytolysis accompanied by internucleosomal DNA fragmentation into multiples of 200 base pairs. Beauvericin has been shown to exhibit varying degrees of toxicity to invertebrates such as *Artemia salina* (Linnaeus) and *Aedes aegypti* (Linnaeus) (Hamill *et al*., 1969; Grove and Pople, 1980) and to invertebrate cell cultures such as *Spodoptera frugiperda* SF-9, a cell line derived from pupal ovaries (Calò *et al*., 2003; Fornelli *et al*., 2004). In addition to invertebrate cell cultures, beauvericin is cytotoxic to a variety of mammalian cell lines such as myeloid cells and cervical carcinoma HeLa cells, at concentrations in the lower micromolar range (Logrieco *et al*., 1996; Logrieco *et al*., 2002a; Calò *et al*., 2004). Due to its significance as a natural contaminant of cereal crops and as an active metabolite of many entomopathogenic fungi, beauvericin levels are of concern to pesticide regulatory agencies such as the Pest Management Regulatory Agency (PMRA), of Health Canada, when considering a biological agent such as *B. bassiana* for insect pest control.

To my knowledge, no current studies are available that correlate whole animal virulence due to the exposure of *B. bassiana* isolates with their respective abilities to produce beauvericin as an indication of virulence. In this study I investigated five *B. bassiana* isolates that exhibited high or low levels of virulence in whole animal assays, for their abilities to produce beauvericin in order to determine if *B. bassiana* isolates can be screened for high levels of whole animal virulence based on beauvericin production. I have also included the commercially available isolate GHA (Emerald BioAgriculture, Lansing, Michigan, USA) to serve as an industry standard.

3.2. Materials and methods

3.2.1. *Fungal toxin and reagents*

Beauvericin (BEA) standard was purchased from Sigma Chemical (St. Louis, Missouri, Lots# 082K1378 and 043K1468). One hundred percent high performance liquid chromatography (HPLC) grade methanol (Fisher Scientific, Ottawa, Ontario) was used to dissolve BEA standard based on Fornelli *et al.* (2004) and Logrieco *et al.* (2002b). Acetonitrile and methanol (HPLC grade) used for HPLC analysis was obtained from Fisher Scientific (Ottawa, Ontario). Water required for the HPLC mobile phase was purified in a Continental Modulab® system (Continental Water Systems, San Antonio, Texas, model# LBPUU101002) for consistency. All reagents used for HPLC analysis were degassed and sterilized using 0.22 µm polyethersulfone (PES) vacuum filtration units (Corning Incorporated, Corning, New York).

3.2.1.1. *Preparation of standard beauvericin for HPLC analysis*

The dilution series was selected based on Fornelli *et al.* (2004) for future use during *in vitro* cell culture studies. To prepare a standard BEA dilution series from 10 mM to 10 µM based on a formula weight of 784, 3.92 mg initially was weighed directly into a sterile 1.5 ml microcentrifuge tube. All subsequent steps were conducted in a biosafety cabinet. Approximately 5 ml of 100% HPLC grade methanol was filtered using a sterile 0.2 µm Nalgene® cellulose acetate syringe filter (Fisher Scientific, Ottawa, Ontario, catalog# 190-2520) then 500 µl was added to the microcentrifuge tube containing the measured quantity of BEA standard and vortexed. The 10 mM solution was then filtered through a sterile 0.22 µm Millex-GV polyvinylidene fluoride (PVDF) syringe filter (Millipore, Billerica, Massachusetts, catalog# SLGV004SL) attached to a sterile BD 1 cc tuberculin syringe (Becton Dickinson & Company, Rutherford, New Jersey) into another sterile 1.5 ml microcentrifuge tube. Once the highest concentration (i.e. 10 mM) BEA solution was prepared, a dilution series from 10 mM to 10 µM was prepared using sterile 100% methanol, sterile microcentrifuge tubes and sterile aerosol barrier micropipette tips to prevent contamination. After the stock concentrations were prepared, 100 µl aliquots of each were dispensed into sterile 0.5 ml microcentrifuge tubes and stored at -20°C until needed.

3.2.2. Beauveria bassiana isolate selection

Beauveria bassiana isolates were selected based on performance in *F. occidentalis* whole animal bioassays (Tables 3.1 and 3.2) and/or variation within *in vitro* growth characteristics.

3.2.2.1. Beauveria bassiana inoculum preparations

Selected isolates were cultured on half-strength (0.5x) Sabouraud dextrose agar (1.5% final agar concentration) [BD (Difco™), Oakville, Ontario] supplemented with 0.5% yeast extract [BD (Difco™), Oakville, Ontario] (0.5xSDAY) in the dark at 23°C ± 2°C for 14 days. Isolates were harvested using a sterilized bacteriological loop and suspended in 2 ml of sterile water according to standard procedures (as per Chapter 2) for a concentration of 10^6 spores/ml beginning with isolate GHA. Isolates were stored at 4°C until needed.

3.2.2.2. *Propagation of Beauveria bassiana isolates*

Propagation of fungal cultures for HPLC analysis was modeled after Peczyńska-Czoch *et al*. (1991) with the following modifications. Sterile 250 ml flasks containing 150 ml of sterile full strength (1x) Sabouraud dextrose broth [BD (Difco™), Oakville, Ontario] supplemented with 1% yeast extract [BD (Difco™), Oakville, Ontario] were each inoculated with 1 ml of 10^6 spores/ml beginning with isolate GHA. Four flasks were inoculated per isolate and were randomly placed in an incubator in the dark at 25°C ± 1°C without agitation for 14 days. This procedure was repeated twice more for 2 of the 5 isolates to account for possible variability in toxin production (Logrieco *et al*., 2002b) and to ensure experimental consistency.

3.2.3. *Extraction of beauvericin*

General extraction protocol followed Peczyńska-Czoch *et al*. (1991) and Logrieco *et al*. (2002b) with the following modifications.

3.2.3.1. *Freeze drying preparation*

After two weeks of incubation, mycelia were harvested and separated from the broth medium. Broth was decanted into 2 l beakers containing a 300 ml solution of 1 part 70% ethanol (23% final concentration) and 2 parts 100% household bleach (3.5% final sodium hypochlorite) for sterilization. Subsequent to removal of broth from all 4 flasks, mycelial mats were transferred to 300 ml clean rounded flat bottom freeze-drying vessels (Labconco, Kansas City, Missouri). All fungal manipulations were done using sterile metal spatulas (Fisherbrand®, Fisher Scientific, Ottawa, Ontario, catalog# 2140110). Once all four mycelial mats from one isolate were transferred, combined mycelia were then washed with 2 volumes (total 125 ml) of sterile distilled water. After washing, the freeze-drying vessels were capped with 70% ethanol-cleaned rubber caps. Once all isolates were transferred to their respective freeze-drying vessels, cultures were shell frozen by submerging the bottom ¾ of the vessel in liquid nitrogen for 10 min. After shell freezing, the vessels were connected to a freeze dryer (Freeze Dry-5®, Labconco, Kansas City, Missouri) at ≤ -50°C (average running temperature of -65°C) and vacuum pressure of ≤ 100 atmospheres (atm) (average running pressure of 50 atm) for 24 h. Vessels were placed in ice baths during initial periods of drying. Respective freeze-drying vessels and caps were weighed prior to initial fungal transfer and after the 24 h freeze drying process to calculate fungal dry weights.

3.2.3.2. Homogenization and extraction

To extract beauvericin, each sample was ground and homogenized using a Polytron® PT-2100 tissue homogenizer and a Polytron® PT-DA 2112/2 EC homogenizing bit (Kinematica, Cincinnati, Ohio) within a biosafety cabinet. Samples were completely ground and homogenized at 11,000 revolutions per min (rpm) progressing up to 19,000 rpm for at least 20 min and were extracted 3 times with 100 ml of methanol (HPLC grade) at 23°C ± 2°C.

Studies conducted by Peczyńska-Czoch *et al.* (1991) and Logrieco *et al.* (2002b) conceivably may have underestimated the mycotoxin(s) synthesized by the fungal isolates investigated. Although Peczyńska-Czoch *et al.* (1991) extracted 3 times with methanol, the homogenization process was not thoroughly described which raises potential questions of proper homogenization procedure as Peczyńska-Czoch *et al.* (1991) reported working with 20 gm of *B. bassiana* mycelia and recovered beauvericin from 3 of the 24 isolates examined. The work of Logrieco *et al.* (2002b) is also questionable. To extract beauvericin, Logrieco *et al.* (2002b) described 10 gm of each *Fusarium* sample was ground and homogenized in an Ultraturrax model T25 basic for 3 min in 50 ml of methanol (HPLC grade) at 13,500 rpm. Based on my observations in working with < 10 gm of fungal dry weight per isolate and a similar tissue homogenizer, it is difficult to achieve complete homogeneity with such a small volume of methanol and homogenizing samples for only 3 min at a relatively low speed of 13,500 rpm.

Methanol extractions were separated from homogenized mycelia by vacuum filtration using a 15 cm fluted Whatman® no. 4 filter paper (Whatman, Florham Park, New Jersey), a 500 ml side-arm flask and a size 58-glass funnel. This process was carried out for the remaining isolates sterilizing the homogenizing bit between each isolate by running the bit in a 4-step sterilization process with 70% ethanol, sterile distilled water, 10% household bleach (0.525% sodium hypochlorite) and sterile distilled water for at least 1 min per step. Once all extractions were complete, each combined extract was vacuum sterilized using a 0.22 μm polyethersulfone (PES) vacuum filtration system (Corning Incorporated, Corning, New York) to ensure complete removal of any ground mycelia that may have bypassed the initial filter.

3.2.3.3. Concentration of samples

The combined sterile extracts were evaporated under reduced pressure at a temperature of 40°C using Heidolph Laborota 4000 (Heidolph Instruments, Schwabach, Germany) and Büchi rotovapor RE120 (Büchi Labortechnik, Flawil, Switzerland) rotary evaporators. Seventy-five ml of extract was added to a 125 ml cleaned rounded flat bottom flask and rotated at 150 rpm until all methanol had evaporated. Forty to forty-five min were required to evaporate all methanol from each isolate. Extract residues were resuspended in a total of 15 ml of methanol (HPLC grade) added to each flask and swirled several times; then the insides of the flask were scraped with customized metal spatulas designed to contact the inner surfaces of the flask. This increased resuspension as insoluble macromolecules such as proteins and carbohydrates may strongly adhere to the inner walls and base of a flask. Some beauvericin molecules may have been sterically hindered due to an insoluble molecular matrix and thus exposure to methanol may have been impeded.

Subsequent to resuspension of each isolate, flask contents were transferred to a sterile 250 ml beaker. Resuspended extracts were degassed/sterilized and purified using a 0.2 µm Nalgene® cellulose acetate syringe filter (Fisher Scientific, Ottawa, Ontario, catalog# 190-2520). Extracts were stored at 4°C to precipitate any additional macromolecules insoluble in methanol at lower temperatures. Prior to HPLC analysis, extracts were filtered again using a 0.2 µm Nalgene® cellulose acetate syringe filter (Fisher Scientific, Ottawa, Ontario, catalog# 190-2520) to remove precipitates. During the HPLC analysis, extracts were maintained at room temperature (23°C ± 2°C) as any further precipitation would be inhibited or delayed at this temperature.

3.2.4. High-performance liquid chromatography analysis

High-performance liquid chromatography analyses were performed as described by Logrieco *et al.* (2002b), with the following modifications. HPLC analyses were performed using a Beckman Coulter System Gold® 168 and a UV-Visible diode array detector (Beckman Coulter Canada, Mississauga, Ontario, system ID# 456880), fitted with a Rheodyne® 7725i injector and a 20 µl loop (Rheodyne, Rohnert Park, California). The software used to conduct the analyses was 32 Karat® (version 5.0, 1998-2001, serial# 021213-09). A Bio-Rad® Hi-Pore Reversed Phase (Bio-Rad Laboratories, Mississauga, Ontario, RP-318, catalog# 125-0551, serial# 416140) C_{18} column (250 mm x 4.6 mm, 5 µm) was also used and equilibrated at 23°C ± 2°C. HPLC conditions included a constant flow at 1.5 ml/min and acetonitrile-water (65:35, volume/volume) as the starting eluting system. The starting ratio was kept constant for 5 min and then linearly modified to 70% acetonitrile in 10 min. After 1 min at 70% acetonitrile, the mobile phase was returned to the starting conditions in 4 min. Beauvericin was detected at 205 nm. Mycotoxin was identified by a comparison of retention times and UV spectra of samples with those of a pure standard. Further confirmation was obtained by coinjecting pure standard with each sample. Beauvericin was quantified by comparing peak areas from samples to a calibration curve of standards.

3.2.4.1. Injection procedure

All samples were filtered through a 0.2 µm Nalgene® cellulose acetate syringe filter (Fisher Scientific, Ottawa, Ontario, catalog# 190-2520) prior to injection (20 µl) onto the column. For each day of analysis, the column was equilibrated at 23°C ± 2°C with the starting conditions of acetonitrile-water (65:35, volume/volume) at a constant flow rate of 1.5 ml/min for 20 min. Subsequently, at least two initial 100% methanol injections were performed to determine column binding efficiency and to ensure a standard baseline chromatogram. Each standard beauvericin concentration was run in triplicate followed by at least two 100% methanol injections to equilibrate the column for the next standard concentration and to ensure no accumulation of beauvericin in the column. To eliminate any residue between standard concentration runs, the injection syringe was washed at least 3 times with 100% methanol. To ensure that cross contamination did not occur within 100% methanol used for washing the syringe and for equilibrating the column, several sterilized/degassed aliquots of 100% methanol were setup using sterile 12 mm x 75 mm polystyrene culture tubes (Simport® Plastics, Beloeil, Quebec) and stored at 23°C ± 2°C. Sample injections were carried out as for standards but with some additions. After the initial two 100% methanol injections, at least one injection of 100 μM standard beauvericin was run to establish daily retention times (due to variation in column binding efficiency) and peak areas (due to changes in concentration because of the volatility of methanol) which were used to confirm beauvericin within samples by coinjection. A fourth injection involved coinjection with 100 μM of standard beauvericin. This concentration of standard beauvericin was selected as it was easily detected and not too concentrated to obscure the sample. Coinjection was done subsequent to triplicate sample injections by first drawing 10 μl of standard beauvericin then 10 μl of sample, mixed via inversion. This approach was taken because prior mixing of sample and standard may have resulted in unequal distribution. Drawing the standard first serves to prevent its contamination and prevents any false detection due to inadequate mixing. This technique of coinjection provides a relatively easy and accurate approach for identification/confirmation. All injections were made using a 100 μl 700 series Hamilton® syringe (Hamilton, Reno, Nevada) in the "inject" position throughout the run to eliminate the appearance of gas bubbles.

3.2.5. Thin layer chromatography analysis

Thin layer chromatography (TLC) analysis was followed after Peczyńska-Czoch *et al.* (1991) with the following modifications, as pure iodide was not available. Twenty to thirty grams of re-sublimed iodine crystals (reagent grade, Amachem Scientific & Chemical) were introduced to a desiccator and heated at ≤ 65°C until the entire desiccator was filled with purple iodine gas. Once equilibrated, alumina-coated TLC plates (EM Science, Darmstadt, Germany) heavily (2 x 5 μl) spotted in triplicate with 10,000 μM, 5,000 μM and 1,000 μM of standard beauvericin dissolved in 100% methanol were placed inside after placing the plates in a solvent system of benzene-ethyl acetate-methanol (19:1:1) for 10 - 15 min and subsequently drying for 10 min. The entire desiccator was then placed in an oven at ≤ 65°C for 40 - 50 min until entire plates were saturated. After complete saturation (i.e. after plates were completely orange with dark orange borders), migration was recorded.

3.2.6. Statistical analysis

All statistical analyses were performed using SAS software version 8.2 (SAS Institute, Cary, North Carolina). Replicate extraction trials and individual replicate runs were subjected to Analysis of Variance (ANOVA) using a Generalized Linear Model. (GLM) procedure. The primary purpose of subjecting the data to ANOVA was to compare if replicate extraction trials were significantly different from the internal control (i.e. isolate GHA), and to determine the relationships between isolates and their respective replicate runs. Differences were considered significant at $p < 0.05$.

3.3. Results

3.3.1. *In vitro beauvericin production from Beauveria bassiana isolates*

Five isolates of *B. bassiana*, varying in whole animal virulence and/or *in vitro* growth characteristics, were selected for toxicological analysis to evaluate *in vitro* beauvericin production. Two of the five isolates were replicated (i.e. replicate extractions) twice more to account for possible variability in toxin production and to ensure experimental consistency. *Beauveria bassiana* methanol extractions were analyzed using HPLC and beauvericin was detected at 205 nm. Beauvericin was identified by a comparison of retention times and UV spectra of samples with those of a pure standard. Further confirmation was obtained by coinjecting pure standard with each sample. Beauvericin was quantified by comparing peak areas from samples to a calibration curve of standards.

Quantitative analyses were based on Figure 3.2 which indicates a linear standard curve generated using a dilution series from 10 mM to 10 μM with an $R^2 = 0.987$. No significant difference was found between replicate injections ($n = 3$) of each standard concentration ($p > 0.2237$, $\alpha = 0.05$).

All 5 *B. bassiana* isolates evaluated in this study produced beauvericin ranging from concentrations of 26.6 μg/g to 2377.5 μg/g (Table 3.3.). No significant difference was found between replicate injections (n = 3) for any evaluation trial (Table 3.4). However, significant differences were found between isolates within all three-evaluation trials (Table 3.4). In comparing isolate GHA across all trials, a significant difference was found between trials (Table 3.5) but no significant difference was found for any replicate injections across trials. Therefore analyses were performed on individual trials.

The chromatogram generated by injecting 100% methanol indicates the degree of non-specific signal ("baseline noise") is shown in Figure 3.3. The chromatogram generated by injecting 1000 μM standard beauvericin indicates the relative retention time and the ratio of signal to the concentration injected is shown in Figure 3.4. The chromatograms generated by injecting extractions of *B. bassiana* isolates to determine the presence or absence of beauvericin and if present, at what concentration are shown in Figures 3.5 – 3.15.

Three concentrations of standard beauvericin (i.e. 10,000 μM, 5,000 μM and 1,000 μM) were used in TLC analysis to determine degree of sensitivity. Although not shown, no migration was evident. In addition, 10,000 μM standard beauvericin was weakly (faintly) visible, 5,000 μM even weaker and 1,000 μM was not visible.

3.4. Discussion

3.4.1. Production of beauvericin by pathogenic isolates of Beauveria bassiana and its relation to whole animal virulence

To evaluate isolates of entomopathogenic fungi for increased efficacy or high levels of virulence, *in vivo* studies using whole animals are conducted. Although such bioassays provide useful information on toxicity towards a target organism, they are laborious and time consuming (Fornelli *et al.*, 2004). To accelerate screening of entomopathogenic fungi for pest management, another approach is to screen based on their metabolites (i.e. mycotoxins).

Mycotoxins play an integral role in the life cycle of many entomopathogenic fungi, including *B. bassiana* (Fuguet and Vey, 2004). In this study, *B. bassiana* isolates earlier evaluated against western flower thrips, *F. occidentalis*, were evaluated for their production of beauvericin to determine if beauvericin could be used as an indicator of whole animal virulence.

To investigate beauvericin production, five isolates of *B. bassiana*, varying in whole animal virulence and/or *in vitro* growth characteristics, were cultured *in vitro* and analyzed for beauvericin production using methanol extractions and HPLC with detection at 205 nm.

No significant difference was found between replicate injections (n = 3) for any evaluation trial (trial 1: $p > 0.2859$, $\alpha = 0.05$; trial 2: $p > 0.2373$, $\alpha = 0.05$; trial 3: $p > 0.3090$, $\alpha = 0.05$). However, significant differences were found between isolates within all three-evaluation trials (trial 1: $p > 0.0001$, $\alpha = 0.05$; trial 2: $p > 0.0010$, $\alpha = 0.05$; trial 3: $p > 0.0015$, $\alpha = 0.05$). To evaluate statistical differences between the three evaluation trials, each individual trial was analyzed using isolate GHA as an internal control. A significant difference was found between trials ($p > 0.0003$, $\alpha = 0.05$) however, no significant difference was found with any replicate injections across trials ($p > 0.4442$, $\alpha = 0.05$). Based on Tukey's statistical grouping evaluation, trials 1 and 2 were similar however evaluation trial 3 was dissimilar. This can be explained by very small amounts of beauvericin binding to the HPLC column leaving behind trace residues that can be detected over time (Li, personal communication). Although HPLC conditions used included a linear increase of acetonitrile in 10 min and held for 1 min at 70% acetonitrile before returning to the starting conditions (designed to elute beauvericin relatively early during the acetonitrile concentration incline), trace quantities may not have been efficiently eluted. It is well known that at least 10 volumes are required to purge the column fully (Li, personal communication). Therefore, based on column dimensions and on the volume formula of a cylinder, $V=\pi r^2 h$, 10 volumes = 41.52 ml which translates into approximately 28 min at 1.5 ml/min to deliver 42 ml. I therefore added at least two 100% methanol injections (at least 40 min; delivering 60 ml; >10 volumes) to purge the column and to reduce the possibility of residues persisting from previous injections. As an additional step, concentrations of 70% - 80% acetonitrile were periodically run through the column for up to 60 min depending on column response. This was to restore binding efficiency by removing strongly adhered molecules which may have accumulated over time. Therefore, not only were absolute beauvericin concentrations used in analyses, but relative concentration ratios as well. This was the reason for fully replicating only two of the five isolates twice more primarily to reveal if the system used in this study is consistent and secondarily, to account for possible variability in toxin production.

All five isolates of *B. bassiana* evaluated in this study produced beauvericin mycotoxin in concentrations ranging from 26.6 µg/g to 2377.5 µg/g (Table 3.3). The range between isolates SR-3 and GHA is 10-fold in magnitude and was consistent across all three-evaluation trials. This indicates that the system employed for extracting, purifying and quantifying beauvericin mycotoxin was consistent.

To my knowledge, this is the first report to indicate that beauvericin is produced by all isolates tested. This is in contrast to Peczyńska-Czoch *et al.* (1991) who showed that among twenty-four isolates surveyed only three produced beauvericin. Based on my study, this discrepancy could result from several factors including media used for fungal propagation, beauvericin extraction technique and the extraction solvent. I used full strength Sabouraud dextrose broth supplemented with 1% yeast extract; this complex medium is commonly used for propagating entomopathogenic fungi (Goettel and Inglis, 1997) as it provides a broad range of growth factors (Madigan *et al.*, 1997). During extraction, complete homogenization of mycelia is critical to yield greater surface area for beauvericin extraction. Peczyńska-Czoch *et al.* (1991) used 20 gm of *B. bassiana* mycelia for each isolate and recovered beauvericin from only 3 of 24 isolates. Furthermore, Logrieco *et al.* (2002b) describe their process of extraction involving 10 gm of *Fusarium* sp. sample ground and homogenized in an Ultraturrax model T25 basic for 3 min at 13,500 rpm with 50 ml of methanol. Based on my observations in working with < 10 gm of fungal dry weight for each isolate and a very similar tissue homogenizer, it seems difficult to achieve complete homogeneity with such a small volume of methanol and homogenizing samples for only 3 min at a relatively low speed of 13,500 rpm. Samples in this study were ground and homogenized starting at 11,000 rpm and progressing up to 19,000 rpm for at least 20 min and were extracted 3 times with 100 ml of methanol. It is conceivable that studies conducted by Peczyńska-Czoch *et al.* (1991)

and Logrieco *et al.* (2002b) underestimated the mycotoxin(s) synthesized by their fungal isolates investigated due to their extraction procedures. Another possibility is ethyl acetate used as an extraction solvent by Peczyńska-Czoch *et al.* (1991). Although beauvericin is freely soluble in most organic solvents (Hamill *et al.*, 1969), some are better than others. Based on my preliminary work, beauvericin standard dissolved instantaneously in 100% methanol. However, it did not completely dissolve in 100% ethanol even after 5 min. Thus, even though Peczyńska-Czoch *et al.* (1991) used methanol for some extraction steps, it is possible that their use of ethyl acetate for others may not have efficiently resuspended beauvericin residues; I did not confirm this with a solubility test.

The probable reason for our discrepancies is difference in chromatographic technique. My results indicate that HPLC is more sensitive for beauvericin analysis than thin-layer chromatography as used by Peczyńska-Czoch *et al.* (1991). Peczyńska-Czoch *et al.* (1991) subjected extracts showing antibacterial activities to TLC analysis and showed that isolation of beauvericin-like products by preparative chromatography yielded compounds with the same antibiotic activity as standard beauvericin (zones of inhibition for beauvericin and other tested compounds = 20 mm at a concentration of 2 mg/ml by the plate diffusion method). For Peczyńska-Czoch *et al.* (1991) to detect beauvericin, high concentrations of the toxin (i.e. beauvericin) were needed. Furthermore, Peczyńska-Czoch *et al.* (1991) exposed TLC plates to iodide vapours as a developing agent to visualize beauvericin. As pure iodide was unavailable, I used re-sublimed iodine crystals in a desiccator and heated the system until the desiccator was filled with purple iodine gas. My results indicated that no migration was evident. In addition, 10,000 μM standard beauvericin was faintly visible, 5,000 μM even weaker and 1,000 μM was not visible. This further suggests that the methods employed by

Peczyńska-Czoch *et al.* (1991) were not as sensitive as mine. Therefore, Peczyńska-Czoch *et al.* (1991) probably underestimated beauvericin production in many of the *B. bassiana* isolates they tested.

Isolate performance against *F. occidentalis* can be ranked as: SR-3 ≈ SR-25 ≈ SR-35 > GHA > SR-11. Isolates SR-25 and SR-35 exhibited high levels of virulence in whole animal bioassays but also produce high levels of beauvericin mycotoxin. Likewise, isolates GHA and SR-11 exhibiting low levels of virulence in such bioassays, will produce low levels of beauvericin mycotoxin. However, isolate SR-3 exhibits high whole animal virulence but produces low levels of beauvericin mycotoxin. Isolates SR-3 and SR-11 produce similar amounts of beauvericin so beauvericin production and whole animal virulence appear to be uncoupled. Moreover, my results suggest a weak correlation between beauvericin production and whole animal virulence: if beauvericin is present in high concentrations, there is a high probability the isolate will also exhibit a high degree of virulence. If beauvericin is present in low concentrations, it may not mean, necessarily that the isolate will exhibit a low degree of virulence.

Some researchers have reported that beauvericin has moderate insecticidal properties (Hamill *et al.*, 1969; Grove and Pople, 1980) while others report it is not involved in entomopathogenicity (Champlin and Grula, 1979). One cannot generalize the role of beauvericin amongst all insect targets. My results indicate that depending on the isolate of *B. bassiana*, beauvericin may or may not be involved as a major virulence factor in terms of western flower thrips pathogenicity. Further studies are needed to determine if this partially dependent relationship between beauvericin and whole animal virulence can be extended to other target insects.

Additional study of beauvericin production by *B. bassiana* is also needed to reveal the prevalence of beauvericin production in additional isolates.

3.5. Summary / Conclusions

Sensitive measurements of beauvericin mycotoxin production by the entomopathogenic fungus, *B. bassiana*, can be evaluated with accuracy and precision by use of HPLC. I found beauvericin to be produced by five isolates of *B. bassiana*. Isolates were selected based on western flower thrips whole animal virulence. An isolate (SR-3) exhibiting one of the highest virulence levels produced low concentrations of beauvericin, indicating that beauvericin production and whole animal virulence are not necessarily coupled. In fact, my results suggest only a weak correlation between beauvericin production and whole animal virulence. When beauvericin is present in high concentrations, there is a greater probability that the isolate will exhibit a high degree of virulence based on 2 conclusive isolates (SR-25 and SR-35). However when beauvericin is present in low concentrations, it does not mean the isolate will exhibit a low degree of virulence. In the latter case, other screening tools such as *in vitro* morphological characteristics may be used in conjunction to avoid laborious and time consuming *in vivo* studies. Nevertheless, beauvericin is important due to its significance as a common metabolite of many phytopathogenic and entomopathogenic fungi, so toxicology studies should be executed to select an isolate having low beauvericin production but high whole animal virulence and when considering registering a biological agent such as *B. bassiana* for insect pest control.

3.6. Tables and figures

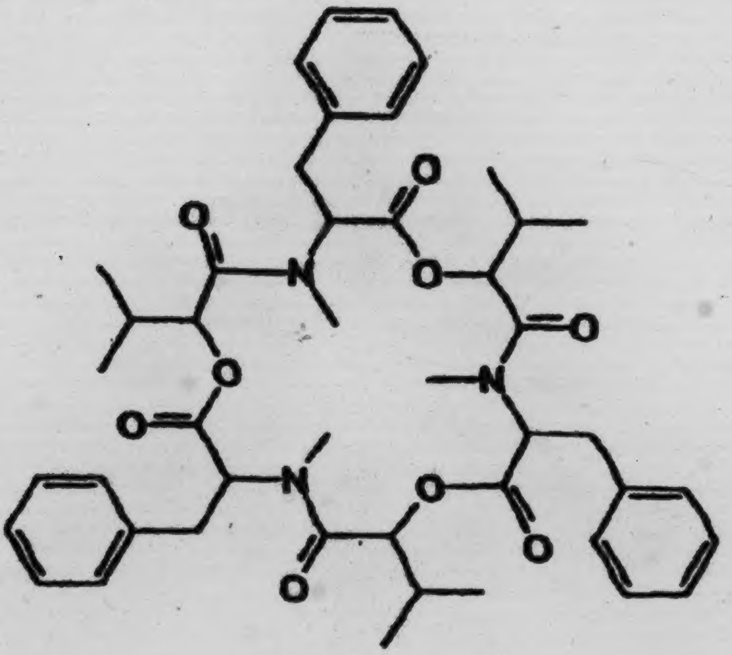

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Figure 3.1. Chemical structure of the beauvericin mycotoxin (Thakur and Smith, 1997).

Table 3.1. *Beauveria bassiana* isolates selected for this study based on whole animal virulence performance in *Frankliniella occidentalis* bioassays.

Identity	Host (order)	Geographic origin	Source
SR-3	Lepidoptera	Canada	B.A. Keddie[b]
SR-11	Hymenoptera	Canada	M. Greif[b]
SR-25	Acari	Canada	M. Greif[b]
SR-35	Hemiptera	Canada	K. Fry[c]
GHA	Coleoptera[a]	USA	Emerald BioAgriculture[d]

[a] According to Becker (2000). Currently, there is a great deal of uncertainty with the original host for isolate GHA (Humber, personal communication).
[b] Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada.
[c] School of Horticulture, Olds College, Olds, Alberta, Canada.
[d] Formerly Mycotech.

Table 3.2. Evaluation of indirect exposure of *Beauveria bassiana* isolates against adult female *Frankliniella occidentalis* by spraying four conidial suspensions ranging from 10^6-10^8 spores/ml and assessing post exposure mortality.

Isolate	LC_{50} (conidia/ml)	± $S.E._{LC50}$ (conidia/ml)	LT_{50} (days)	± $S.E._{LT50}$ (days)
SR-3[a]	2.85E+07	1.66E+06	4.73	0.11
SR-11[a]	8.28E+09	8.07E+09	N/D[c]	N/D[c]
SR-25[a]	3.52E+07	1.12E+07	4.94	0.34
SR-35[a]	2.67E+07	4.30E+06	4.80	0.20
GHA[b]	2.48E+09	1.31E+09	5.95	0.22

[a] Pooled trials (n = 3). Mean LC_{50} and LT_{50} values reported.
[b] Pooled trials (n = 6). Mean LC_{50} and LT_{50} values reported.
[c] Non-discernable response. Actual response value = 2.11E+13.

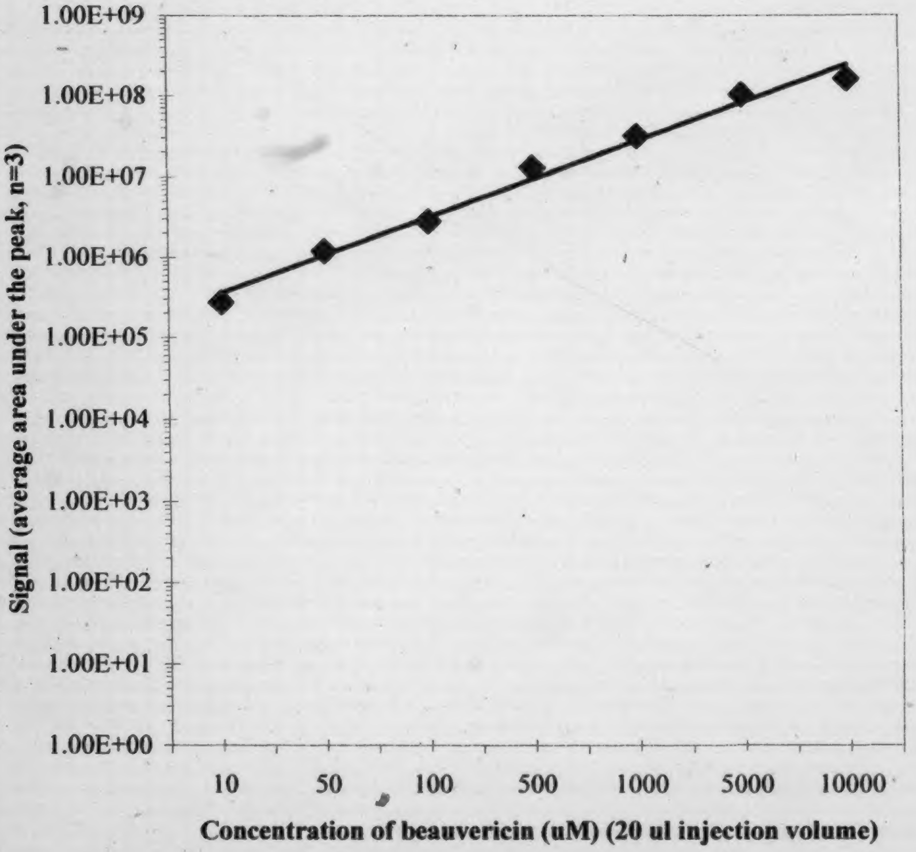

Figure 3.2. Standard curve representing increasing concentrations of beauvericin standard in relation to signal generated at 205 nm ($R^2 = 0.987$).

Table 3.3. *Beauveria bassiana* isolates selected based on whole animal bioassays against *Frankliniella occidentalis*.

Evaluation trial	Isolate	Beauvericin concentration (ug/g)
1[a]	SR-3	91.9 a
	SR-11	26.6 a
	GHA	828.3 b
	SR-35	1821.6 c
	SR-25	2377.5 d
2[a]	SR-3	112.4 a
	GHA	1041.2 b
3[a]	SR-3	150.3 a
	GHA	1719.0 b

[a] Means with the same letter are not significantly different at $p < 0.05$ using Tukey means separation test.

Table 3.4. Type III sum of squares (SS) across each individual evaluation trial to determine if any isolates or replicates within a trial are significantly different ($\alpha = 0.05$).

Evaluation trial	Source	DF	F-Value	P-Value
1	Isolate	4	396.14	0.0001
	Rep	2	1.47	0.2859
2	Isolate	1	982.42	0.0010
	Rep	2	3.21	0.2373
3	Isolate	1	680.19	0.0015
	Rep	2	2.24	0.3090

Table 3.5. Type III sum of squares (SS) across all evaluation trials (n = 3) to determine if any trials or replicates (within and among) are statistically different by assessing an internal standard namely isolate GHA ($\alpha = 0.05$).

Source	DF	F-Value	P-Value
Trial	2	111.34	0.0003
Rep	2	1.00	0.4442

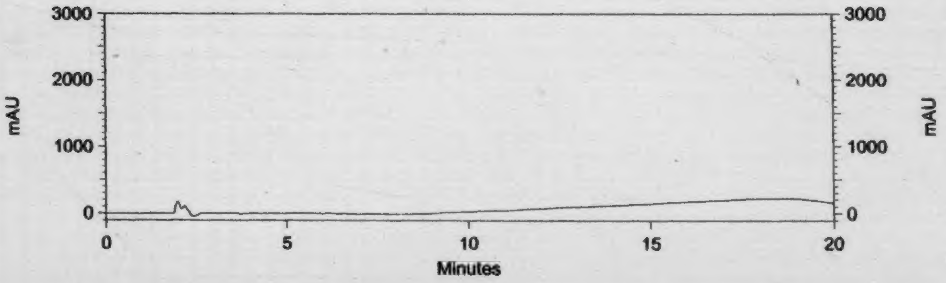

Figure 3.3. Chromatogram representing signal generated at 205 nm by injecting 20 µl of 100% methanol (HPLC grade) ("baseline noise"). Signal measured in milliabsorbance units (mAU).

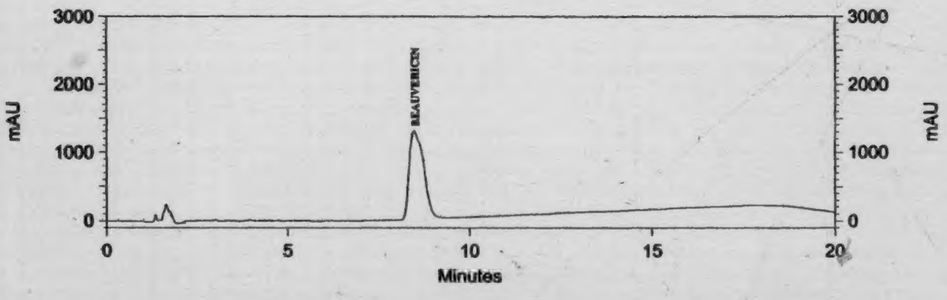

Figure 3.4. Chromatogram representing signal generated at 205 nm by injecting 20 µl of 1000 µM standard beauvericin. Signal measured in milliabsorbance units (mAU).

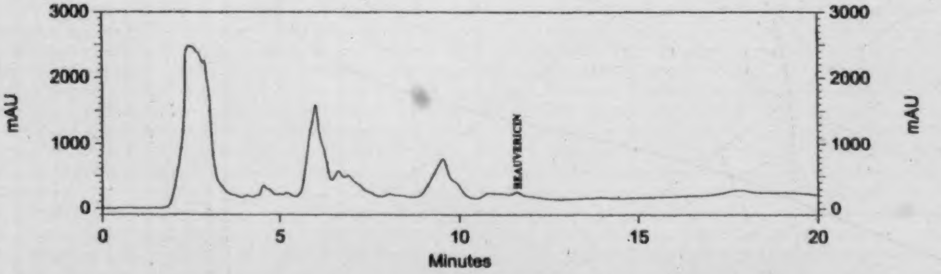

Figure 3.5. Chromatogram representing signal generated at 205 nm by injecting 20 μl of GHA methanol extraction. Signal measured in milliabsorbance units (mAU).

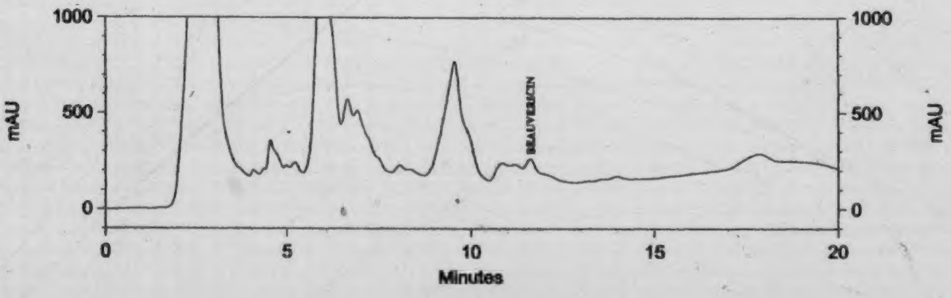

Figure 3.6. Focused chromatogram representing signal generated at 205 nm by injecting 20 μl of GHA methanol extraction. Signal measured in milliabsorbance units (mAU).

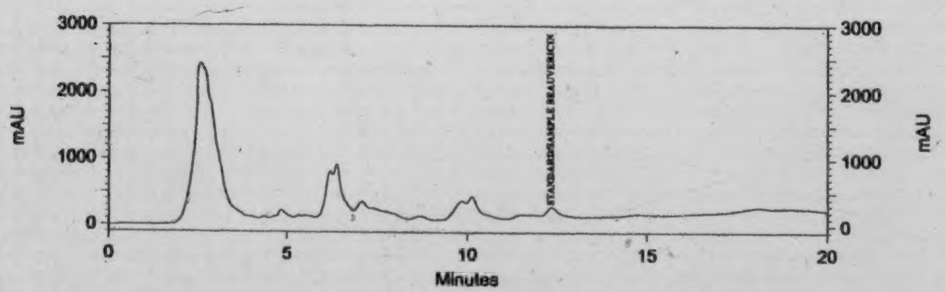

Figure 3.7. Chromatogram representing signal generated at 205 nm by coinjecting 10 μl of GHA methanol extraction and 10 μl of 100 μM standard beauvericin. Signal measured in milliabsorbance units (mAU).

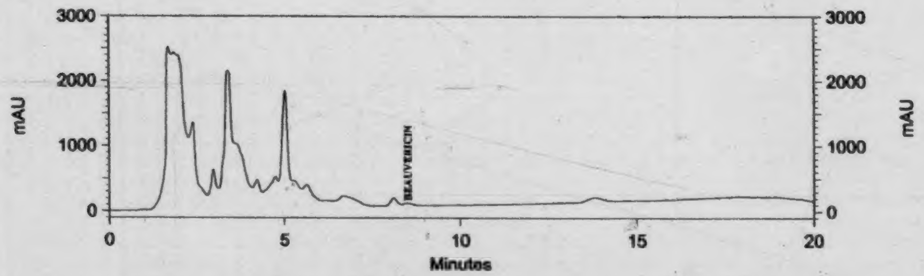

Figure 3.8. Chromatogram representing signal generated at 205 nm by injecting 20 µl of SR-3 methanol extraction. Signal measured in milliabsorbance units (mAU).

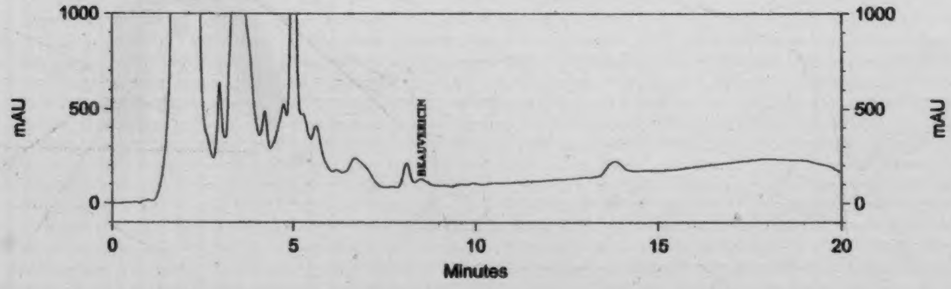

Figure 3.9. Focused chromatogram representing signal generated at 205 nm by injecting 20 µl of SR-3 methanol extraction. Signal measured in milliabsorbance units (mAU).

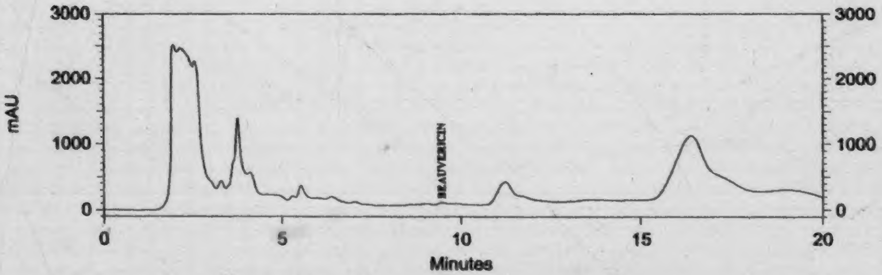

Figure 3.10. Chromatogram representing signal generated at 205 nm by injecting 20 µl of SR-11 methanol extraction. Signal measured in milliabsorbance units (mAU).

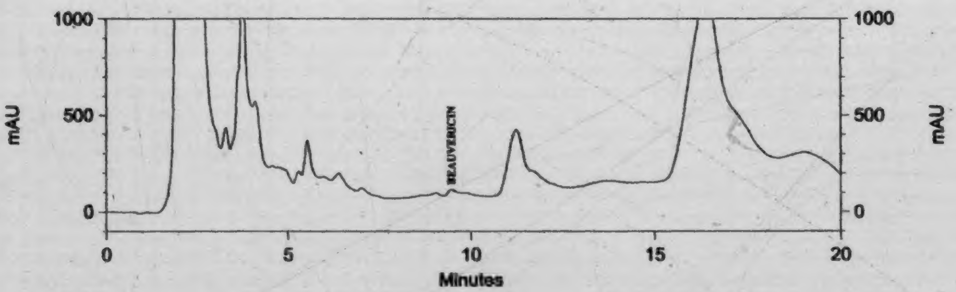

Figure 3.11. Focused chromatogram representing signal generated at 205 nm by injecting 20 µl of SR-11 methanol extraction. Signal measured in milliabsorbance units (mAU).

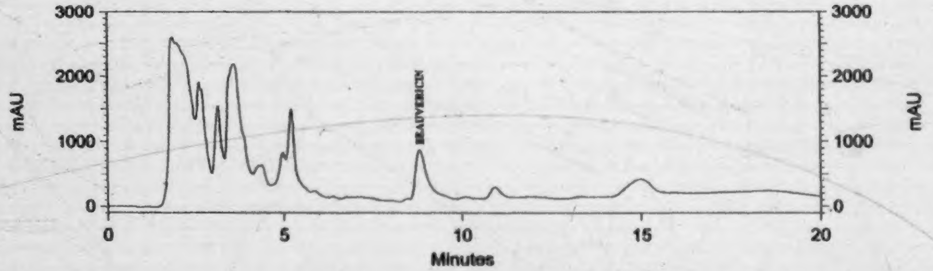

Figure 3.12. Chromatogram representing signal generated at 205 nm by injecting 20 μl of SR-25 methanol extraction. Signal measured in milliabsorbance units (mAU).

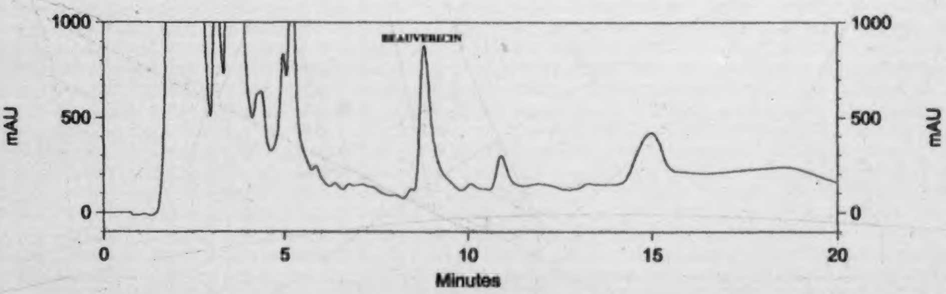

Figure 3.13. Focused chromatogram representing signal generated at 205 nm by injecting 20 μl of SR-25 methanol extraction. Signal measured in milliabsorbance units (mAU).

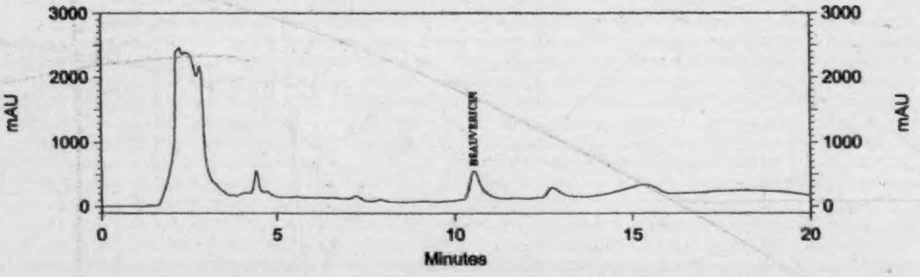

Figure 3.14. Chromatogram representing signal generated at 205 nm by injecting 20 µl of SR-35 methanol extraction. Signal measured in milliabsorbance units (mAU).

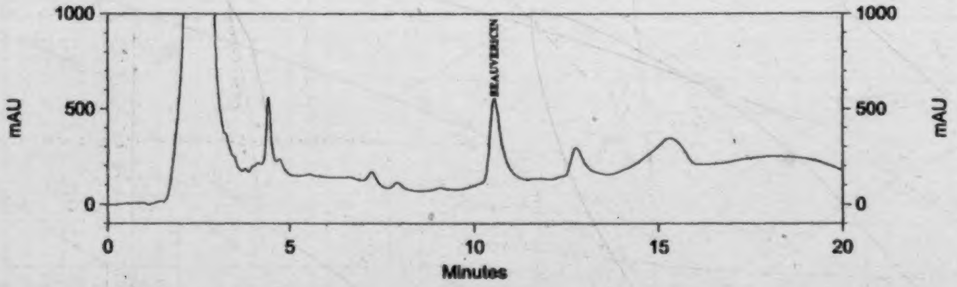

Figure 3.15. Focused chromatogram representing signal generated at 205 nm by injecting 20 µl of SR-35 methanol extraction. Signal measured in milliabsorbance units (mAU).

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Chapter 4. Cytotoxicity of beauvericin to a Hemipteran (*Bemisia argentifolii*) cell line (Btb(Ba)97 Hunter-Polston)

4.1. Introduction

The conventional approach to *in vivo* study of entomopathogenic fungi and their metabolites provides useful information on their influence on insect fitness (Fornelli *et al.*, 2004). For example when exposed to isolates of *Beauveria bassiana* (Balsamo) Vuillemin that exhibit a range of virulence, fitness parameters such as survivorship of adult thrips (e.g. *Frankliniella occidentalis* Pergande) can be used to select an effective isolate(s) for an integrated pest management (IPM) program. Although *in vivo* studies using whole insects are useful in providing toxicological information on a target organism, such tests are laborious and time consuming (Fornelli *et al.*, 2004).

To avoid these difficulties, Fornelli *et al.* (2004), suggested that *in vitro* assays are preferable in toxicity assessment of various classes of environmental contaminants including fungal metabolites. This approach significantly reduces evaluation time and can provide information about the mode of action of the toxicant (Fornelli *et al.*, 2004). In addition, *in vitro* cytotoxicity tests are useful for determining dose- and time-dependent cytotoxicity, as measured by the potential of a compound to induce cell death in different cell types (Eisenbrand *et al.*, 2002). *In vitro* toxicity assessment has increased in recent years as it is usually less expensive, more quantitative, reproducible and efficient than are *in vivo* studies (Fornelli *et al.*, 2004). Furthermore, *in vitro* analysis allows one (1) to control the microenvironment surrounding the cells (e.g. pH, temperature, osmotic pressure, and oxygen and carbon dioxide tension) which is difficult or impractical to control in a localized and precise manner *in vivo*; (2) to independently measure various physiological elements and with limited interference from other molecules present *in vivo*; (3) to expose a reagent directly and at a lower and more defined concentration to the cell with less reagent than for an *in vivo* injection, where 90% is lost to excretion and distribution to other tissues; and (4) to have a homogeneous cell population where cells have similar needs and can be optimized for efficient growth (Freshney, 2000).

Unfortunately, a previous attempt to establish a thysanopteran cell line by Hunter and Hsu (1996) was unsuccessful. I attempted to establish such a cell line based on Hunter and Hsu (1996) but also without success. Instead, I selected a whitefly, *Bemisia argentifolii* (Bellows and Perring), cell line as a model system for an *in vivo* comparison as this insect is the most closely related to Thysanoptera (Grimaldi and Engel, 2005) of any, readily available, cell lines. Furthermore whiteflies are important pests and are also susceptible to entomopathogens.

In this study, I evaluated the toxic effects induced by the fungal metabolite, beauvericin (BEA) *in vitro* using an alternative invertebrate model, the hemipteran *B. argentifolii* cell line Btb(Ba)97 Hunter-Polston. My primary focus is to evaluate the sensitivity of Btb(Ba)97 Hunter-Polston cells to beauvericin and to determine whether this approach could be used to screen *Beauveria bassiana* isolates.

Beauvericin was initially reported to be synthesized by entomopathogenic fungi such as *B. bassiana* (Hamill *et al*., 1969) and *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Bernardini *et al*., 1975; Logrieco *et al*., 2002). Subsequently, it has been detected in numerous entomopathogenic and phytopathogenic isolates of *Fusarium* species. (Gupta *et al*., 1991; Logrieco *et al*., 1993a; Logrieco *et al*., 1993b; Moretti *et al*., 1995). Due to its significance as a natural contaminant of cereal crops and a metabolite of many biocontrol fungi, beauvericin levels are of concern to pesticide regulatory agencies such as the Pest Management and Regulatory Agency (PMRA). As a requirement of the PMRA, toxicity studies using *in vitro* models must be conducted to register a pesticide for use in Canada (Lucarotti, personal communication).

Previous researchers have described insect cell lines used to test toxins (Quiot *et al.*, 1985; Logrieco *et al.*, 1996; Fornelli *et al.*, 2004). These studies address the toxicity induced by fungal metabolites such as destruxins, fusaproliferin, nivalenol, deoxynivalenol, and fumonisin B1. Insect cell lines are a reliable *in vitro* evaluation of mycotoxins and a few studies have included beauvericin (Calò *et al.*, 2003; Fornelli *et al.*, 2004) using lepidopteran cell lines such as *Spodoptera frugiperda* (SF-9). Using the hemipteran *Bemisia argentifolii* (Btb(Ba)97 Hunter-Polston) cell line to evaluate the cytotoxic effects of beauvericin is a novel approach. I have also developed a novel *in situ* approach to assess cell viability using Trypan blue dye exclusion.

At least two assays, MTT-colourimetric and Trypan blue dye exclusion, are commonly used to indicate viability in toxicological studies both characterized by low cost and quick response (Fornelli *et al.*, 2004). Both viability assays have distinct and specific cellular targets, mitochondrial function and membrane permeability changes, respectively. Fornelli *et al.* (2004) found no significant difference between IC_{50} (50% inhibiting concentration) and CC_{50} (50% cytotoxic concentration) values obtained by the MTT test and Trypan blue dye exclusion for 16 fungal metabolites having different chemical structures and biological activity. I selected Trypan blue for this study for materials and equipment efficiency. Trypan blue dye exclusion assay is a cell viability assay based on the ability of live cells to exclude Trypan blue; its uptake indicates irreversible membrane damage preceding cell death. Trypan blue dye exclusion is used routinely to quantify the reduction in percentage of viable cells and the cytotoxic effects of toxins.

4.2. Materials and methods

4.2.1. Cell culture

Whitefly (*B. argentifolii*) (Bellows *et al.*, 1994) cells obtained from embryonic tissues (designated 'Btb(Ba)97 Hunter-Polston', Hunter and Polston, 2001) were maintained at 27°C ± 1°C in 4 ml/T-25 flasks (Corning Incorporated, Corning, New York) of HyQ® SFX-Insect™ medium (HyClone, Logan, Utah, catalog# SH30278.02, several Lot#s were used) supplemented with 6% heat-inactivated fetal bovine serum (FBS) (volume/volume) (Sigma Chemical, St. Louis, Missouri, catalog# F-0643, Lot# 119H84201) (Figures 4.1 and 4.2). Three-week-old cultures in early stationary phase (typical cell density, 1.7 x 10^6 cells/ml with an average cell viability of 86%) were split every 7 days with a starting density of 6.8 x 10^5 cells/ml. In this system, cell-doubling time is approximately 3 days; this result is consistent with Hunter and Polston (2001).

4.2.1.1. Cell passage

Due to the strong adherent properties of Btb(Ba)97 Hunter-Polston cells, cultures were passaged using 1.5 ml 0.25% porcine trypsin (Sigma Chemical, St. Louis, Missouri, catalog# T4674, Lot# 41K2360). Porcine trypsin was purchased as a 10x stock solution and aliquoted to be stored at -20°C. Prior to use, the trypsin was diluted 10-fold using

0.22 µm filtered Rinaldini's balanced salt solution (RBSS) (Rinaldini, 1959), aliquoted and stored at -20°C. For each passage, all medium was removed from a 21-day old flask (cell density, 1.7 x 10^6 cells/ml, slightly greater than a confluent monolayer) and 1.5 ml of 0.25% trypsin was added. Flasks containing trypsin were incubated for 4 min with gentle rocking. After 4 min, 2 ml of fresh HyQ SFX medium with 6% FBS was added. With the reaction quenched, a sterile 5 ml borosilicate pipette was used to release the cells by gently rinsing the flask surface 7-8 times with the trypsin/medium solution. Released cells were transferred to a sterile 15 ml centrifuge tube (Corning Incorporated, Corning, New York) and centrifuged at 1000 - 1500 revolutions per minute (rpm) for 2 min using an IEC clinical centrifuge. Following centrifugation, the supernatant was removed and the cells resuspended in 5 ml of fresh HyQ SFX medium with 6% FBS. Once the cells were resuspended, 1 ml was added to a sterile T-25 flask. Before adding medium, each flask was gently rocked to spread the cells followed by addition of 2.5 ml of fresh medium. After passage, cell density was approximately 6.8 x 10^5 cells/ml. Newly passaged T-25 flasks were maintained weekly by removing old medium and adding 4 ml of fresh HyQ SFX medium with 6% FBS beginning 7 days post passage. Cells were passaged every 7 days using 21-day source flasks.

4.2.2. Beauvericin

Beauvericin (cyclo-[D-∝-hydrossyisovaleryl-L-*N*-methyl-phenylalanyl]$_3$) (BEA) standard was purchased from Sigma Chemical (St. Louis, Missouri, Lot# 082K1378). Purity was confirmed using HPLC techniques (as per Chapter 3) to ensure the standard contained no trace contaminate(s) which could cause downstream interference. One hundred percent HPLC grade methanol (Fisher Scientific, Ottawa, Ontario) was used to dissolve BEA standard based on Fornelli *et al.* (2004) and preliminary tests were run using other diluents (Rajput, data not shown).

4.2.2.1. Toxin preparation

A dilution series was selected based on Fornelli *et al.* (2004) and the result of preliminary studies (Rajput, data not shown). A standard BEA dilution series ranging from 10 mM to 1 μM was prepared based on a formula weight of 784 as per Chapter 3 with the following modifications. Once the highest concentration (i.e. 10 mM) BEA solution was prepared, a dilution series from 10 mM to 1 μM was prepared using sterile 100% methanol, sterile microcentrifuge tubes and sterile aerosol barrier micropipette tips to prevent contamination. After the stock concentrations were prepared, 7 μl aliquots of each were dispensed into sterile 0.5 ml microcentrifuge tubes and 343 μl of HyQ SFX medium containing 6% FBS was added (a 1:50 dilution). Control methanol was also diluted 1:50 with HyQ SFX medium containing 6% FBS. All aliquots were stored at -20°C until needed.

4.2.2.2. Confirmation of purity of standard beauvericin using HPLC techniques

High-performance liquid chromatography (HPLC) analysis was performed as per Chapter 3 to confirm purity (Figure 4.3).

4.2.3. Cell culture and treatments

Cell culture treatments were similar to those of Fornelli *et al.* (2004) with the following modifications. Cultures in early stationary phase (21 days, typical cell density of 1.7 x 10^6 cells/ml with an average cell viability of 86%) were trypsinized to seed in 96-well microtitration plates with a starting density of 1.9 - 2.9 x 10^7 cells/ml (corrected for viability). Following trypsin treatment, the following changes were made. After centrifugation and removal of the supernatant, cells were gently resuspended in 10 ml of fresh HyQ SFX with 6% FBS medium. Once resuspended, cells were poured into a sterile 100 x 15 mm petri dish to accommodate an 8-channel micropipetter. Sterile aerosol barrier tips were used to prevent contamination. Three rows of 11 wells were seeded in a sterile flat bottom 96-well microtitration plate (Costar®, Corning Incorporated, Corning, New York) for a triplicate assay, with each well receiving 200 μl of cell suspension. In addition, 800 μl of cell suspension was aliquoted into a sterile microcentrifuge tube for enumeration and viability assessments using a Reichert Neubauer Improved Bright-Line haemocytometer (Reichert Incorporated, Depew, New York) and 0.2 μm filtered 0.4% Trypan blue solution (Sigma Chemical, St. Louis, Missouri, Lot# 103K2350) at a ratio of 1:2 (volume/volume).

Plates were double sealed with parafilm and incubated at 27°C ± 1°C for 48 h before testing. After incubation, 100 μl was removed from each well and replaced with either 100 μl of HyQ SFX with 6% FBS medium (a control media-only treatment), or control methanol amended with medium or test solutions amended with medium. Test solutions were added at a ratio of 1:2 (volume/volume). The final concentration of solvent in the cultures assayed was 1%. Thus, the final ratio of the test solutions was added at 1:100 (volume/volume) as described by Fornelli *et al.* (2004). Amending all test solutions and control methanol with medium prevented changes to the prepared concentrations due to possible evaporation of solvent and allowed for better suspension of toxin. Adding 2 µl of concentrated test solution directly to a well as described by Fornelli *et al.* (2004) may cause cell trauma leading to their premature death. Mortality observed by using such a small concentrated volume may be an overestimate. Plates were double sealed with parafilm and incubated at 27°C ± 1°C for 48 h.

4.2.4. Bioassays

4.2.4.1. Trypan blue dye exclusion

Trypan blue dye exclusion was conducted 48 h after incubation of test solutions. With each replicate series, 180 µl of medium was removed from each well and 20 µl of 0.2 µm filtered 0.2% Trypan blue solution (diluted with RBSS) was added to the sides of each well to prevent a void volume in the centre of the well. This reduced potential trauma to cells and the possibility of false positives. Following addition of Trypan blue, each well was viewed and photographed at 100x using an Olympus® inverted microscope (Olympus America, Melville, New York) and a Nikon Coolpix® 775 digital camera (Nikon Canada, Mississauga, Ontario). A viable cell will exclude an acid dye such as Trypan blue, while its uptake indicates irreversible membrane damage preceding cell death (Fornelli *et al.*, 2004).

4.2.4.1.1. *In situ viability assessments using digital imagery*

To assess cell viability *in situ* using Trypan blue, four images were taken of each well beginning with the controls in a clockwise fashion starting with top left position (TL), top right position (TR), bottom right position (BR) and bottom left position (BL). This procedure was performed on the remaining replicates with total Trypan blue incubation times recorded for each replicate. Images were imported into Microsoft Powerpoint® at a standardized size of 17.3736 cm (height) x 23.1648 cm (width) centered on a landscape slide measuring 19.05 cm (height) x 25.4 cm (width). Each slide was labeled with its microculture well designation and its image position (e.g. B1(TL) refers to well B1 top left position). For each image, a 1 x 1 cm grid was overlaid to facilitate random sub-sampling (Figure 4.4). For each treatment, at least 500 cells were counted to assess viability. The ratio of live to dead cells (cell viability) was determined, standard curves were prepared and 50% cytotoxic concentrations (CC_{50}; i.e. the concentrations of BEA causing a 50% decrease in cell viability) were derived (Fornelli *et al.* 2004). This *in situ* approach to assess viability results in increased accuracy and precision in comparison to sub-sampling from each treated microculture of cells (i.e. a tissue culture plate well), mixing it with a solution of Trypan blue and then assessing viability using a haemocytometer (Fornelli 2004, personal communication).

Results are presented as arithmetic means of homogeneous experiments corrected for solvent control mortality with standard deviations. The assays were repeated four times independently and each BEA concentration or controls were tested in triplicate each time.

4.2.4.2. *Statistical analysis*

All statistical analyses were performed using SAS software version 8.2 (SAS Institute, Cary, North Carolina). Replicate trials and individual replicate assays (i.e. both within and among bioassay trials) were subjected to Analysis of Variance (ANOVA) using a Generalized Linear Model (GLM) procedure. The primary purpose of subjecting the data to ANOVA was to compare the entire replicated experiment trials (n = 4) and the replicates within (n = 3) to determine if the trials and replicates could be pooled to derive a more precise CC_{50}. Differences were considered significant at $p < 0.05$.

4.3. Results

4.3.1. *Cytotoxicity to whitefly Btb(Ba)97 Hunter-Polston cells*

Early stationary phase cultures of *B. argentifolii* embryonic Btb(Ba)97 Hunter-Polston cells exposed to medium-only showed high viability (98% ± (standard deviation; s.d.) 0.7%; n = 12) and exhibited normal cytological features (Figure 4.5). In addition, cells exposed to solvent alone (1% volume/volume) showed normal viability (98% ± s.d. 1.2%; n = 12) and exhibited normal cytological features (Figure 4.6). Cells exposed beauvericin test solutions, at concentrations ranging from 0.01 μM to 100 μM, showed variable degrees of viability and cytotoxic effects as shown in Figures 4.7 – 4.9.

No significant difference was found between values obtained among the four replicate evaluation trials ($p > 0.4780$, $\alpha = 0.05$) or between individual replicates within each trial ($p > 0.9490$, $\alpha = 0.05$) (Figure 4.10). Therefore the results were pooled, generating a dose-response curve obtained by Trypan blue dye exclusion (Figure 4.11). At lower concentrations of beauvericin (i.e. 0.01 μM, 0.05 μM and 0.1 μM) there was a slight decrease in viability (12% ± 5.0% (n = 12), 10% ± 6.4% (n = 12) and 13% ± 9.7% (n = 12), respectively). However no apparent cytological effects were visible at 0.01 μM and 0.05 μM. Cytological effects, such as loss of adherent properties, became noticeable at concentrations ranging from 0.1 μM to 0.5 μM. Increasing beauvericin concentrations to 0.5 μM, 1 μM and 5 μM led to a definite decline of 23% ± 5.6% (n = 12), 27% ± 11.0% (n = 12) and 37% ± 10.8% (n = 12) in viability, respectively; with even a sharper decline of 57% ± 7.5% (n = 12) and 89% ± 1.0% (n = 12) at a concentration of 10 μM and 50 μM, respectively (Figures 4.7 and 4.8). At a concentration of 100 μM, cell viability was severely reduced (95% ± 0.8% (n = 12)) (Figures 4.9 and 4.11). Loss of adherent properties became evident at concentrations ranging from 5 μM to 10 μM. Signs of trauma, such as cell lysis, became more pronounced with concentrations greater than 10 μM. After exposing cells to the mycotoxin beauvericin, the CC_{50} after a 48 h incubation was interpolated as 7.9 μM ± 2.0 μM (Figure 4.11). A standard deviation was calculated for CC_{50} values reported by pooling the replicates (n = 3) of each trial (n = 4) and generating dose-response curves from which CC_{50} values were interpolated from each trial generating a standard deviation of the arithmetic mean.

4.4. Discussion

4.4.1. Effects of beauvericin on Bemisia argentifolii Btb(Ba)97 Hunter-Polston cell line

Cytotoxicity of beauvericin to an immortalized whitefly cell line (Btb(Ba)97 Hunter-Polston) composed predominantly of fibroblast-like cells derived from *B.*

argentifolii embryonic tissues (Hunter and Polston, 2001), was tested to determine if this cell line is sensitive to beauvericin in a dose-dependent manner and can be used as a screening tool for virulent *B. bassiana* isolates.

Cells in early stationary phase were exposed to a range of beauvericin concentrations along with the appropriate controls (48 h post seeding) and the resulting viability was assessed 48 h post exposure via Trypan blue dye exclusion and digitally photographed. A routine approach of assessing viability using Trypan blue dye exclusion was modified to yield a more accurate and precise measurement. Some researchers have sub-sampled from each treated microculture of cells (i.e. a tissue culture plate well), mixing it with a solution of Trypan blue and then assessing the viability using a haemocytometer (Fornelli 2004, personal communication). With this approach, a greater proportion of nonviable cells are selected as highly adherent cell types lose their adherent properties as they are exposed to increasing concentrations of certain fungal toxins. This *in situ* approach of removing a specific quantity of medium and adding Trypan blue directly to the microculture of cells and subsequently digitally capturing the response allows for an unbiased assessment of cell viability and a photographic record of each assay.

No significant difference was found between the values obtained among the four replicate evaluation trials ($p > 0.4780$, $\alpha = 0.05$) or between the individual replicates within each trial ($p > 0.9490$, $\alpha = 0.05$) (Figure 4.10). Therefore the results were pooled, generating a dose-response curve obtained by Trypan blue dye exclusion (Figure 4.11). At lower concentrations of beauvericin (i.e. 0.01 μM, 0.05 μM and 0.1 μM) there is a slight decrease in viability (12% ± 5.0% (n = 12), 10% ± 6.4% (n = 12) and 13% ± 9.7%

(n = 12), respectively). However no apparent cytological effects were visible at either 0.01 μM or 0.05 μM. Cytological effects, such as loss of adherent properties, became noticeable at concentrations ranging from 0.1 μM to 0.5 μM. Increasing beauvericin concentrations to 0.5 μM, 1 μM and 5 μM led to a definite decline of 23% ± 5.6% (n = 12), 27% ± 11.0% (n = 12) and 37% ± 10.8% (n = 12) in viability, respectively; with even a sharper decline of 57% ± 7.5% (n = 12) and 89% ± 1.0% (n = 12) at a concentration of 10 μM and 50 μM, respectively (Figures 4.7 and 4.8). At a concentration of 100 μM, cell viability was severely reduced (95% ± 0.8% (n = 12)) (Figure 4.9). Loss of adherent properties became quite evident at concentrations ranging from 5 μM to 10 μM. Signs of trauma, such as cell lyses, became more pronounced with concentrations greater than 10 μM. After exposing Btb(Ba)97 Hunter-Polston cells to the mycotoxin beauvericin, the CC_{50} after a 48 h incubation was interpolated as 7.9 μM ± 2.0 μM (Figure 4.11).

This is the first report on the cytotoxic effects of beauvericin on a *B. argentifolii* cell line Btb(Ba)97 Hunter-Polston. Results obtained with this *in vitro* model show that the Btb(Ba)97 Hunter-Polston cell line derived from *B. argentifolii* embryonic tissue is sensitive to beauvericin in a dose-dependent fashion. Preliminary results also indicated that beauvericin affects the cells in a time-dependent fashion (Rajput, data not shown).

My results are fully comparable with those of other investigators such as Fornelli *et al*. (2004) in their investigation of fungal metabolites on *Spodoptera frugiperda* cell line SF-9. Fornelli *et al*. (2004) reported the toxicity of beauvericin on SF-9 cells as a CC_{50} value of 3.0 μM ± 1.0 μM also assessed by Trypan blue dye exclusion. The CC_{50} derived from Btb(Ba)97 Hunter-Polston cell line was 7.9 μM ± 2.0 μM which is in the same order of magnitude. The discrepancy shows that the cell line derived from the envelope of pupal ovaries of *S. frugiperda* is more sensitive to beauvericin and this difference may relate to their derivation from insects in distantly related insect orders or to the different origin of the tissue (Fornelli *et al.* 2004). Another possibility for increased sensitivity in SF-9 cells may be due to their incorporation of antibiotics (200 mM penicillin-streptomycin-neomycin solution, volume/volume) within the TNM-FH cell culture medium used by Fornelli *et al.* (2004). Lynn (1996) reported that if bacterial or fungal contamination occurs, incorporating antibiotics within a cell culture medium will mask contamination until resistance develops which may take weeks or months. A basal degree of stress is placed on cells in culture, as they must metabolize antimicrobial and/or antifungal agents and compete with bacteria or fungal cells, if present. Without using antibiotics in insect culture media, real assurance of sterility can be guaranteed, as any contamination will be quickly apparent. Furthermore, without antibiotics incorporated in the media, cells are more representative of their source tissues since such cells are not normally exposed to such agents. Hence, when evaluating compounds in an *in vitro* system, it is best to omit antibiotics as they may cause unnecessary interference and costs.

The cytotoxicity results I obtained after exposure to beauvericin also agrees with Calò *et al.* (2003); in that the CC_{50}, after exposing SF-9 cell cultures for 24 h to beauvericin, was estimated as approximately 10 μM. Exposing cultures to beauvericin for 72 h resulted in an approximate CC_{50} of 2.5 μM. Therefore, beauvericin affects cells in a dose- and time-dependent fashion. My CC_{50} value of 7.9 μM ± 2.0 μM obtained from Btb(Ba)97 Hunter-Polston cultures exposed to beauvericin for 48 h is consistent with the values reported by Calò *et al.* (2003).

Extending to mammalian *in vitro* systems, there are several cytotoxicity studies of fungal metabolites on mammalian cell lines (Ojcius *et al.*, 1991; Visconti *et al.*, 1991; Hanelt *et al.*, 1994; Gutleb *et al.*, 2002; Calò *et al.*, 2004). Focusing on beauvericin toxicity, similar results were observed in both the insect cell line used in this study and in mammalian cell lines (Logrieco *et al.*, 2002; Calò *et al.*, 2004). Calò *et al.* (2004) generated results from two human myeloid cells lines, with a similar dose- and time-dependent decrease in viability and CC_{50} values of ~30 μM for the monocytoid U-937 cells and ~15 μM for the undifferentiated promyelocytic HL-60 cells were reported. Calò *et al.* (2004) further agree that their results are consistent with their earlier work (2003) using the lepidopteran cell line SF-9. Therefore it seems that beauvericin affects invertebrate and mammalian cells in a similar manner (Calò *et al.*, 2004).

Although insect cell lines are used extensively as a model to study the mechanism of action and effects of fungal metabolites (Quiot *et al.*, 1985; Dumas *et al.*, 1994, 1996; Liu *et al.*, 1996; Logrieco *et al.*, 1996; Calò *et al.*, 2003; Fornelli *et al.*, 2004), it is important to relate *in vitro* studies to *in vivo* studies (Eisenbrand *et al.*, 2002). For example, fungal metabolites can depress growth and cause changes in mortality, fertility, egg viability and metamorphosis (Dowd, 1992; Mulé *et al.*, 1992; Pangrahi, 1993; Vey *et al.*, 2001; Fornelli *et al.*, 2004).

Ganassi *et al.* (2002) exposed aphids (*Schizaphis graminum* Rondani) to 25 μg/ml in 0.5% methanol (31.89 μM) of beauvericin by ingestion through leaves of their host plant that adversely affected their fitness: fecundity was significantly reduced in members of the second and third generation with an increasing number of aborted embryos. Using histological techniques, Ganassi *et al.* (2002) detected an interaction between beauvericin and nucleic acids of the bacterial endosymbiont *Buchnera aphidicola*. I showed that exposing Btb(Ba)97 Hunter-Polston cells to concentrations of beauvericin ranging from 10 µM – 50 µM for 48 h, decreased cell viability from 57% ± 7.5% – 89% ± 1.0%, respectively. In comparing with the results obtained from Ganassi *et al*. (2002) and results obtained in my study, the whitefly Btb(Ba)97 Hunter-Polston cell line provides a more sensitive (as the interactions that take place *in vitro* are minimal in comparison with *in vivo* interactions) indication of *in vivo* toxicity at least for some other hemipterans such as aphids. Fornelli *et al*. (2004) reported that SF-9 cells were more sensitive to certain mycotoxins than was the whole crustacean *Artemia salina* (Linnaeus). In examining beauvericin, the data obtained with SF-9 cells was comparable to that obtained with *A. salina* (Fornelli *et al*., 2004).

Fornelli *et al*. (2004) also compared the toxicity ranking of beauvericin to that of enniatin using the SF-9 cell line model with injection studies (Grove and Pople, 1980) on adult blowflies *Calliphora erytrocephala* (Meigen) and larvae of the mosquito *Aedes aegypti* (Linnaeus). Using SF-9 cells, Fornelli *et al*. (2004) confirmed the results of *in vivo* studies where enniatin cytotoxicity was lower than that of beauvericin as observed by Grove and Pople (1980). The similarity of these results indicates that *in vitro* studies provide a reliable estimate of *in vivo* interactions, for at least some mycotoxins.

In vitro studies can rapidly screen for toxicity of microbial metabolites and may support extrapolation to *in vivo* risks for humans (Eisenbrand *et al*., 2002). When screening *B. bassiana* isolates for insect pest management, the Btb(Ba)97 Hunter-Polston cell line can be used with a minor calculation adjustment to evaluate *in vitro* impacts. Based on previous research (Chapter 3), beauvericin is produced by all 5 isolates tested in varying amounts. By determining the quantity of beauvericin that a particular isolate synthesizes, a division correction factor of 100, based on the concentration series used in this study, will allow one to interpolate or extrapolate the percent mortality induced on Btb(Ba)97 Hunter-Polston cells in other studies. A practical implication of knowing the quantity of beauvericin produced by an isolate and relating it to the results of *in vitro* studies, will be to address key requirements of concern to the PMRA when registering a biological control agent for use in Canada.

Although *in vitro* assays are useful as they are usually less costly, more quantitative, reproducible and efficient than are *in vivo* studies (Fornelli *et al*., 2004), further *in vivo* studies are needed to understand the effects of beauvericin in a more complex and dynamic environment. This is important to consider because fungal metabolites, such as beauvericin, will encounter cells organized into tissues or organs in whole animals where the number of interactions between cells is greater. Despite the limitations of *in vitro* systems, they provide an initial approach to address fundamental research questions in a reliable, economical and time efficient manner.

4.5. Summary / Conclusions

In vitro model systems such as insect cell lines provide more precision in controlling the physiochemical microenvironment and in establishing relatively constant physiological conditions for reliable and increased efficiency in toxicity comparison to *in vivo* studies using whole animals. In this study, the toxicity of beauvericin produced by some entomopathogenic and phytopathogenic fungi was evaluated on a whitefly *B. argentifolii* (Btb(Ba)97 Hunter-Polston) cell line by using Trypan blue dye exclusion *in situ*. Using a cell line derived from embryonic tissue, I found it to be sensitive to the fungal metabolite beauvericin in a dose-dependent and time-dependent fashion. After exposing the cells to beauvericin, I interpreted the CC_{50} after a 48 h incubation to be 7.9 μM ± 2.0 μM comparable to results of other researchers using other cell lines.

4.6. Tables and figures

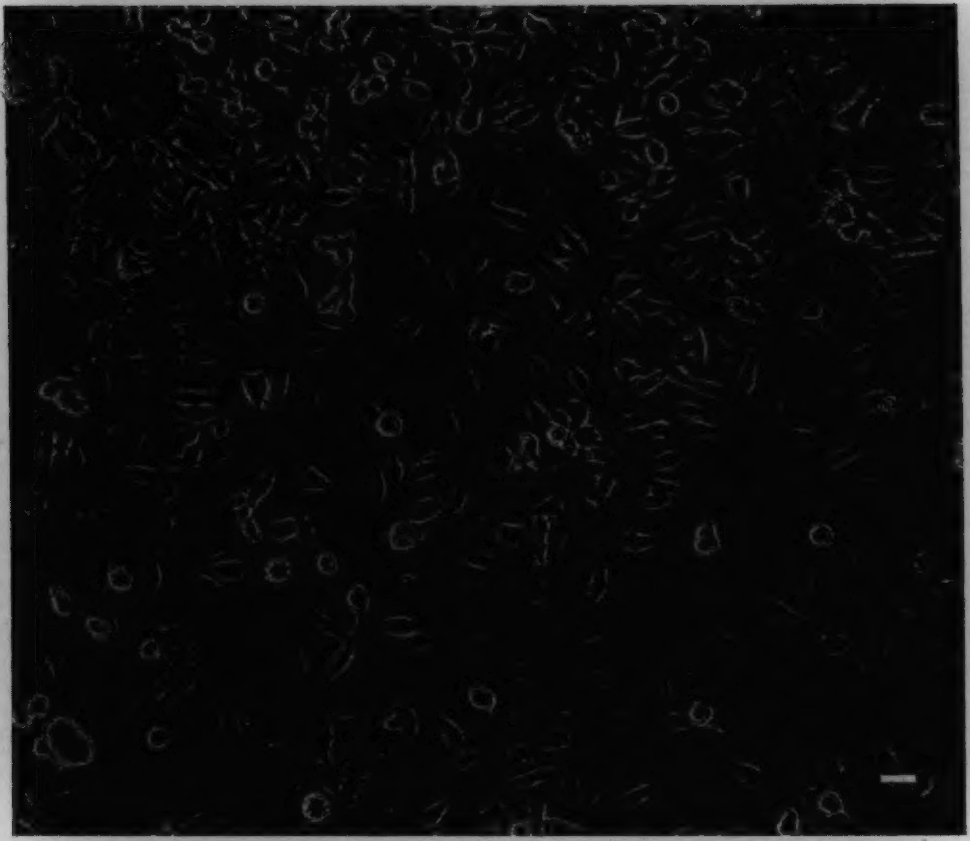

Figure 4.1. Light micrograph of a cultured cell monolayer of Btb(Ba)97 Hunter-Polston cell line derived from embryonic cells of *Bemisia argentifolii*. Image of fibroblast and epithelial cell monolayer. Scale bar = 100μM.

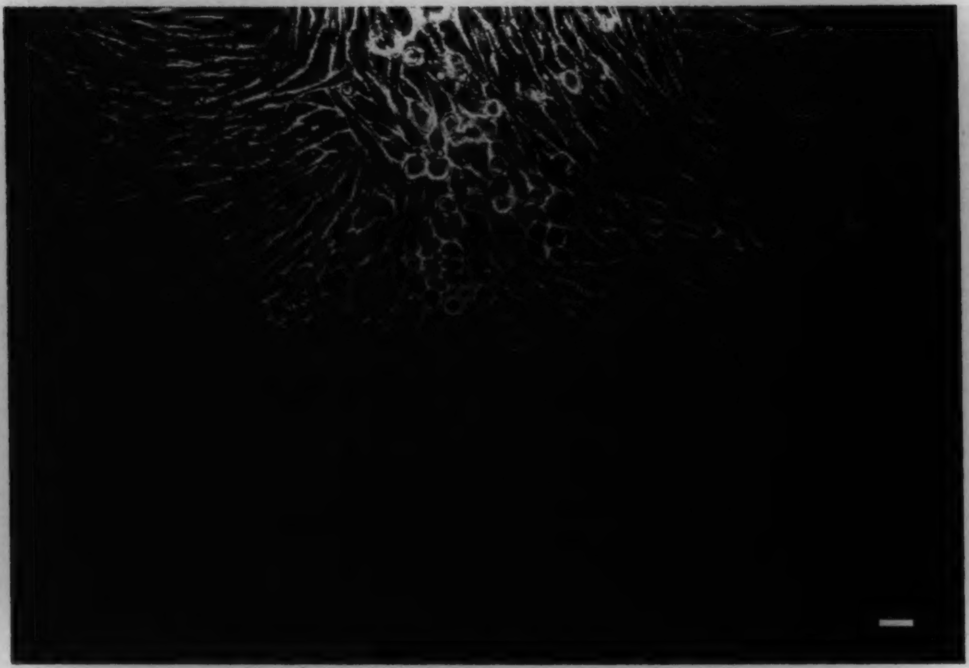

Figure 4.2. Light micrograph of *Bemisia argentifolii* predominant fibroblasts in culture. Image of fibroblast and epithelial cell monolayer. Scale bar = 100µM.

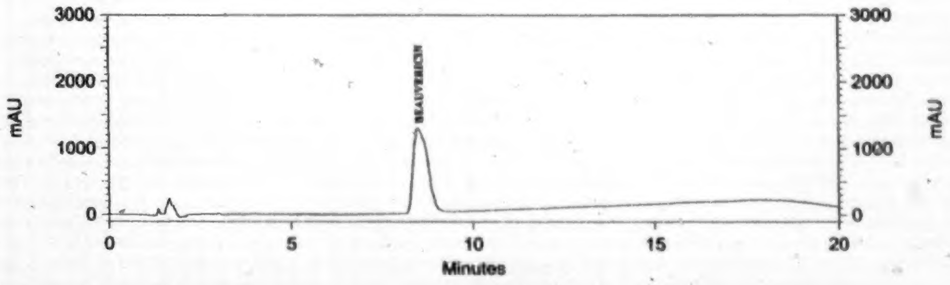

Figure 4.3. Chromatogram representing signal generated at 205 nm by injecting 20 µl of 1000 µM standard beauvericin. Signal measured in milliabsorbance units (mAU).

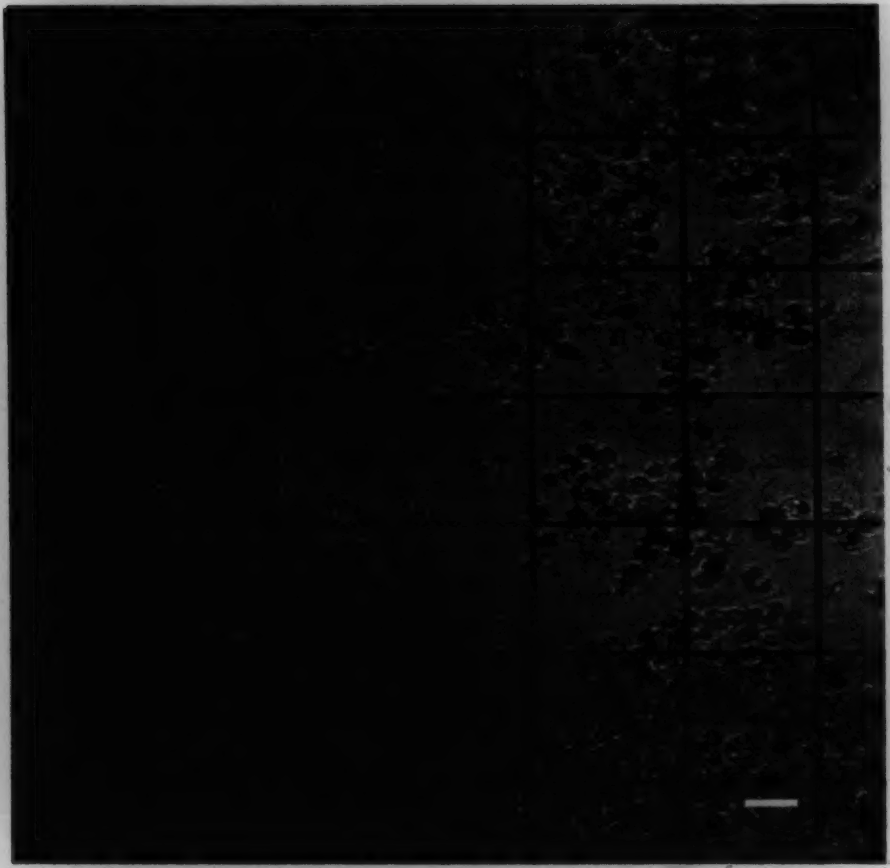

Figure 4.4. A micrograph of one of four digitally captured fields of view taken of treated Btb(Ba)97 Hunter-Polston cells with an 1 cm x 1 cm grid overlay to allow for random sub-sampling. At least 500 cells were counted per treatment to assess cell viability. Scale bar = 200μM

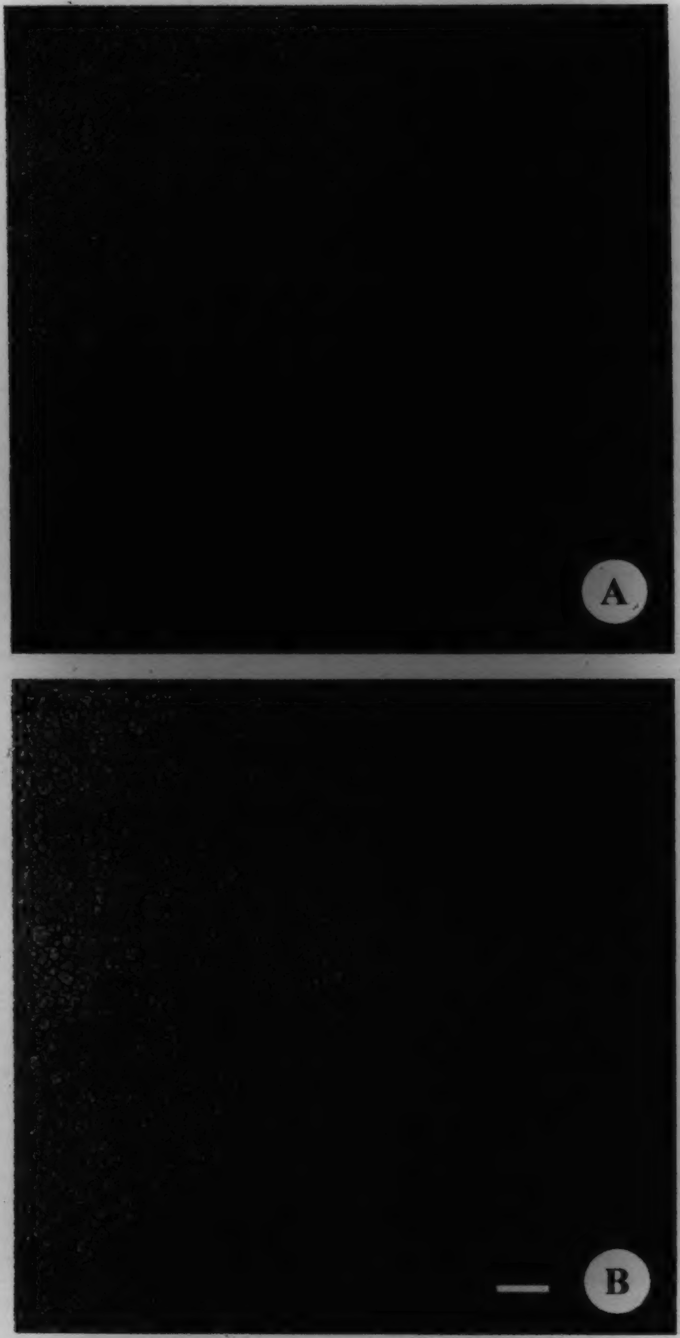

Figure 4.5. Light micrograph of *Bemisia argentifolii* Btb(Ba)97 Hunter-Polston cells exposed to negative control HyQ SFX medium with 6% FBS ("neat") for 48 hours. Post incubation, cell viability was assessed with 0.2% Trypan blue solution. Cell death is indicated by uptake of the dye (arrows). (A) and (B) two negative control treatments from two different assays both from the bottom right (BR) position of the microtitration well as indicated by the greater density of dye in the BR corner. Scale bar = 200μM.

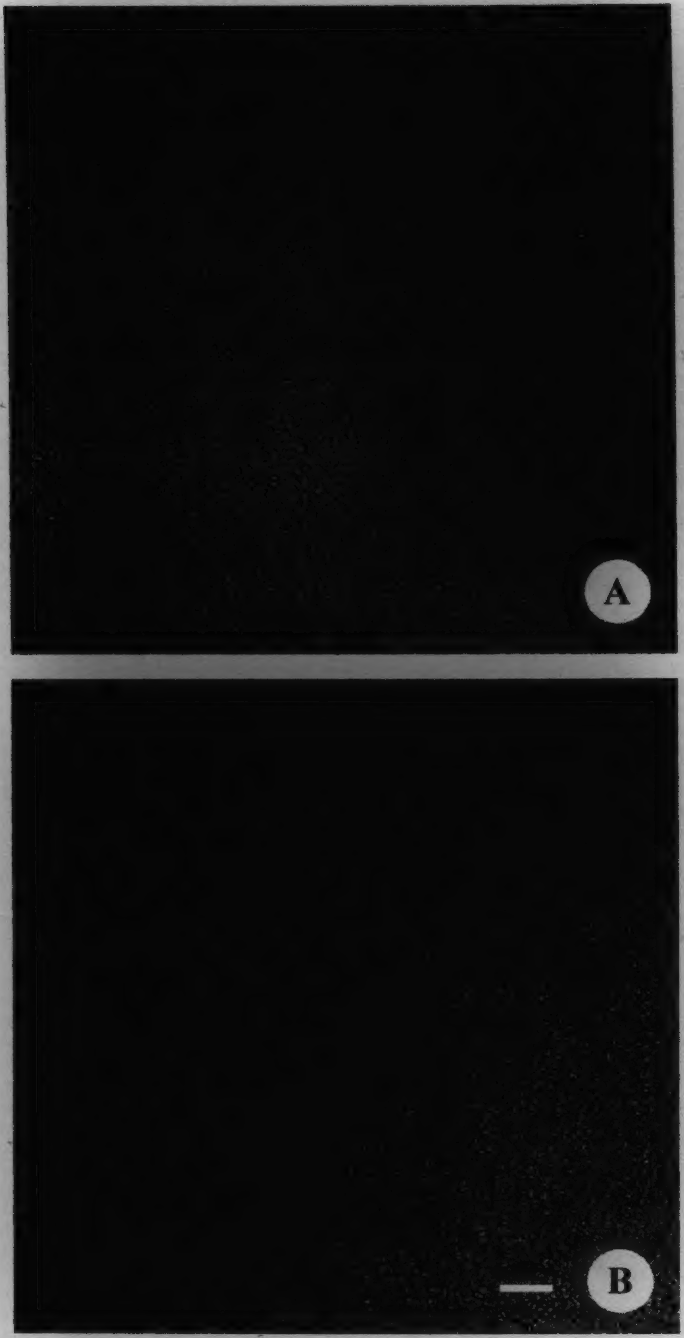

Figure 4.6. Light micrograph of *Bemisia argentifolii* Btb(Ba)97 Hunter-Polston cells exposed to control 1% HPLC grade methanol amended with HyQ SFX medium with 6% FBS for 48 hours. Post incubation, cell viability was assessed with 0.2% Trypan blue solution. Cell death is indicated by uptake of the dye (arrows). (A) and (B) two negative methanol control treatments from two different assays: (a) TR position (b) TL position. Scale bar = 200μM.

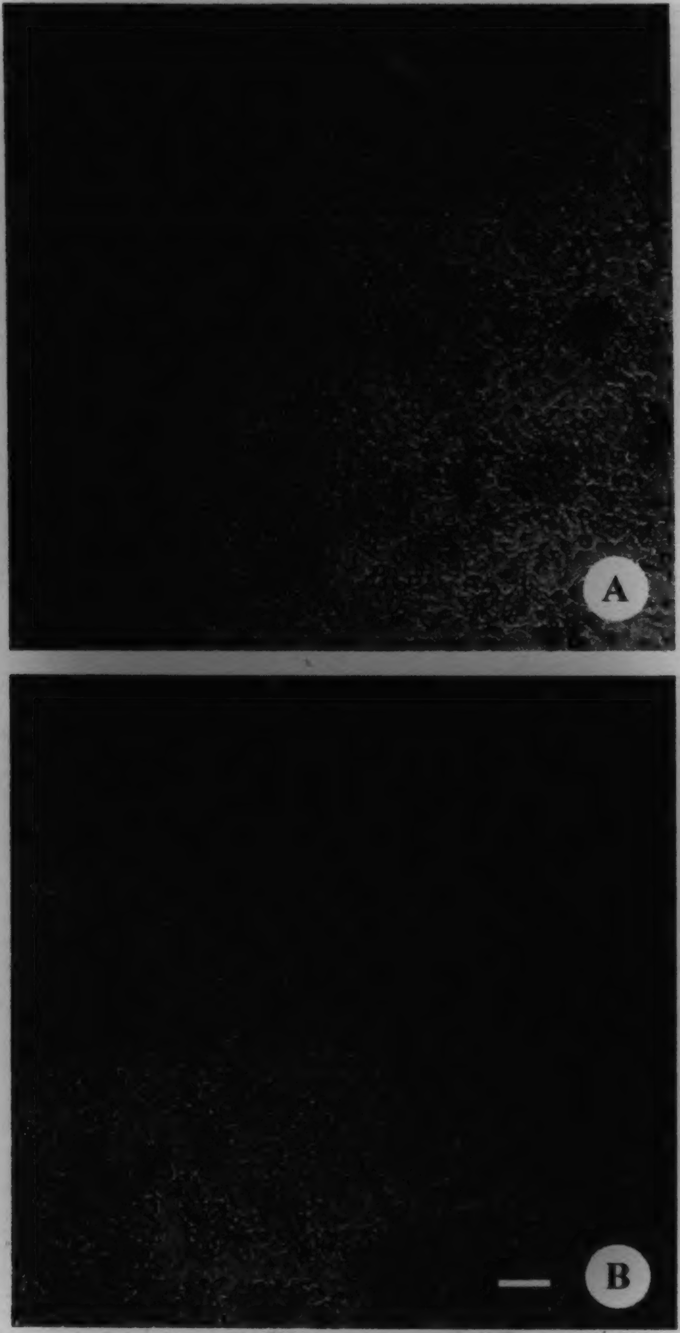

Figure 4.7. Light micrograph of *Bemisia argentifolii* Btb(Ba)97 Hunter-Polston cells exposed to 1 μM standard beauvericin dissolved in methanol amended with HyQ SFX medium with 6% FBS for 48 hours. Post incubation, cell viability was assessed with 0.2% Trypan blue solution. Cell death is indicated by uptake of the dye (arrows). (A) and (B) two 1 μM treatments from two different assays: (a) TL position (b) TR position. Scale bar = 200μM.

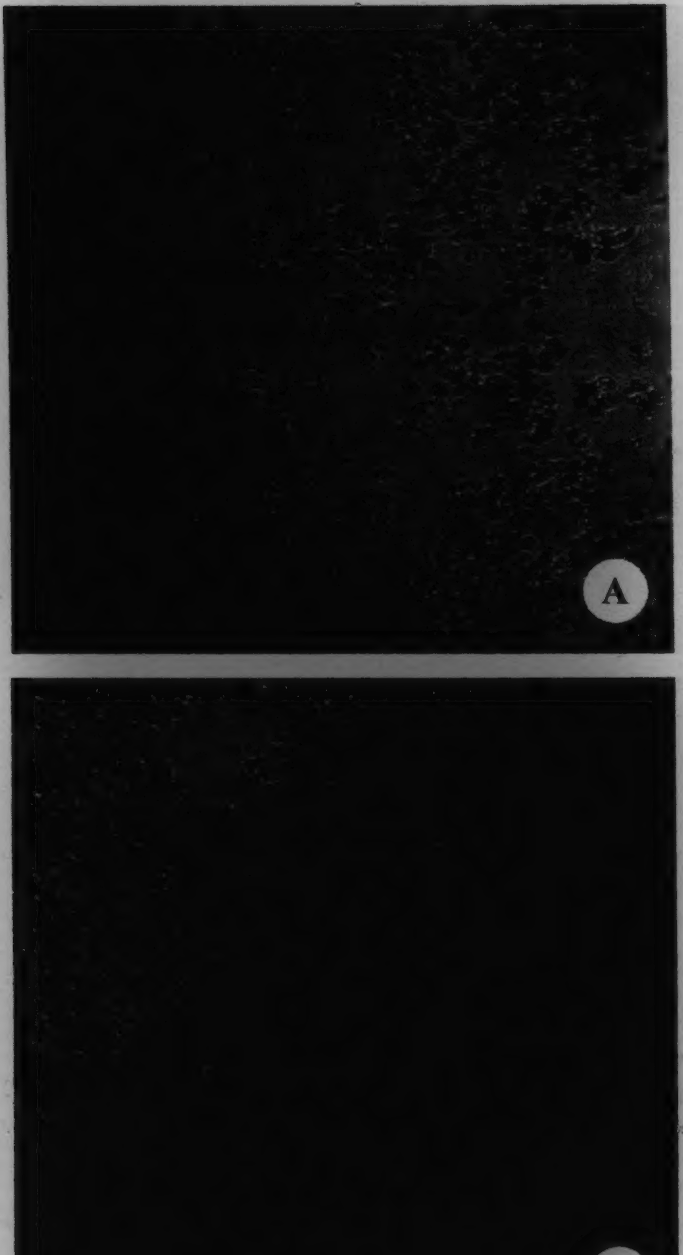

Figure 4.8. Light micrograph of *Bemisia argentifolii* Btb(Ba)97 Hunter-Polston cells exposed to 10 µM standard beauvericin dissolved in methanol amended with HyQ SFX medium with 6% FBS for 48 hours. Post incubation, cell viability was assessed with 0.2% Trypan blue solution. Cell death is indicated by uptake of the dye (arrows). (A) and (B) two 10 µM treatment from two different assays: (a) BL position (b) BR position. Scale bar = 200µM.

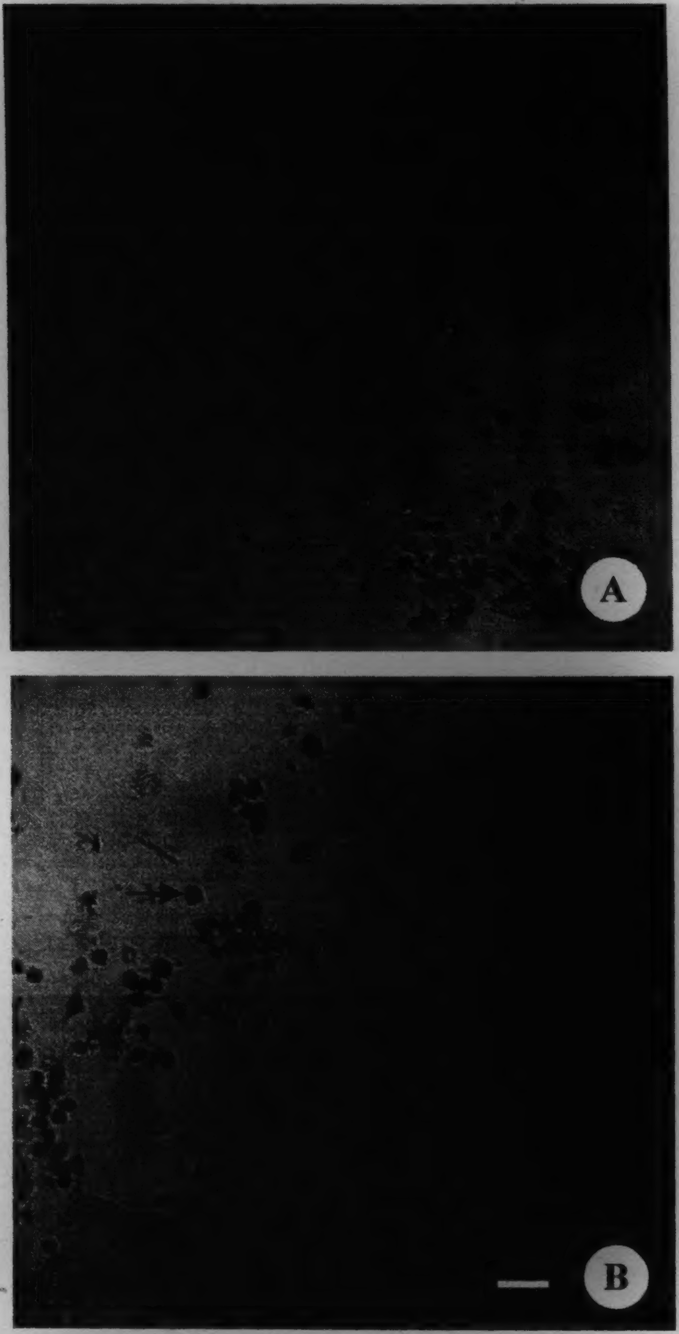

Figure 4.9. Light micrograph of *Bemisia argentifolii* Btb(Ba)97 Hunter-Polston cells exposed to 100 μM standard beauvericin dissolved in methanol amended with HyQ SFX medium with 6% FBS for 48 hours. Post incubation, cell viability was assessed with 0.2% Trypan blue solution. Cell death is indicated by uptake of the dye (arrows). (A) and (B) two 100 μM treatment from two different assays: (a) TL position (b) BR position. Scale bar = 200μM.

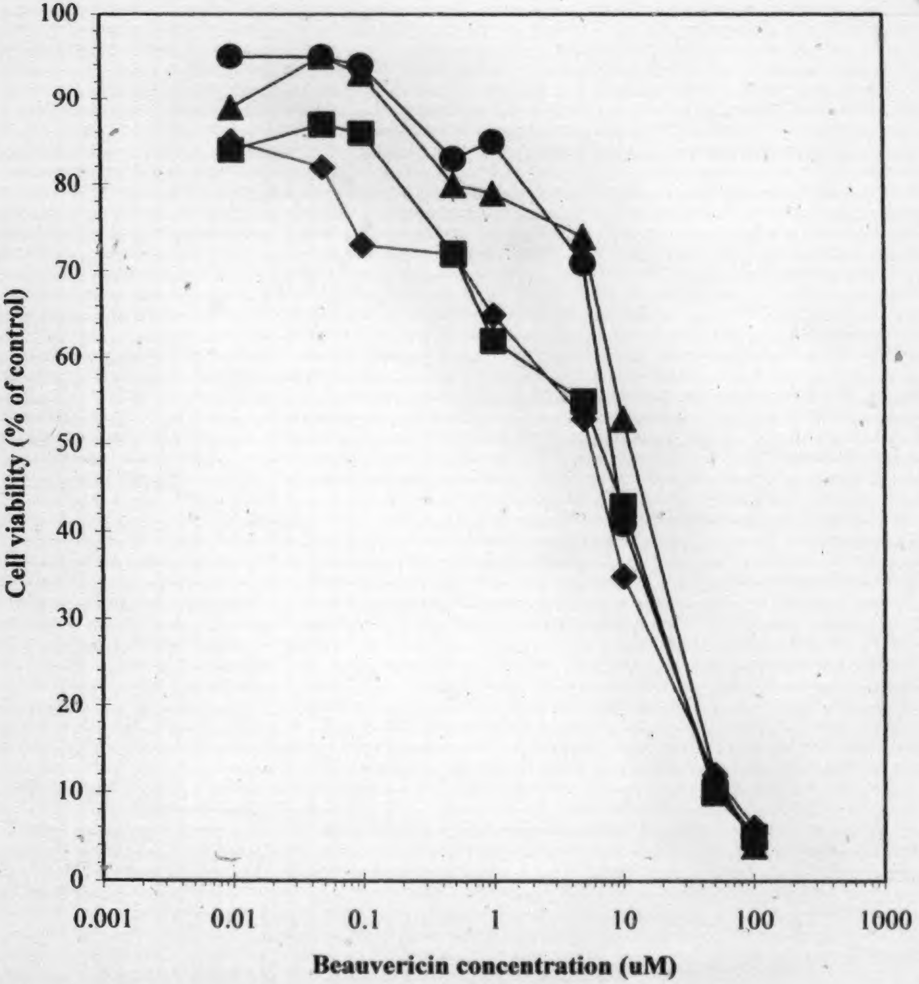

Figure 4.10. Log scale of pooled dose-response based on the mean cell viabilities obtained with standard beauvericin 48 hours post exposure to whitefly cell line Btb(Ba)97 Hunter-Polston and evaluated by 0.2% Trypan blue dye exclusion. [–◆–, Trial 8 (n = 3); –■–, Trial 9 (n = 3); –▲–, Trial 10 (n = 3); –●–, Trial 11 (n = 3)].

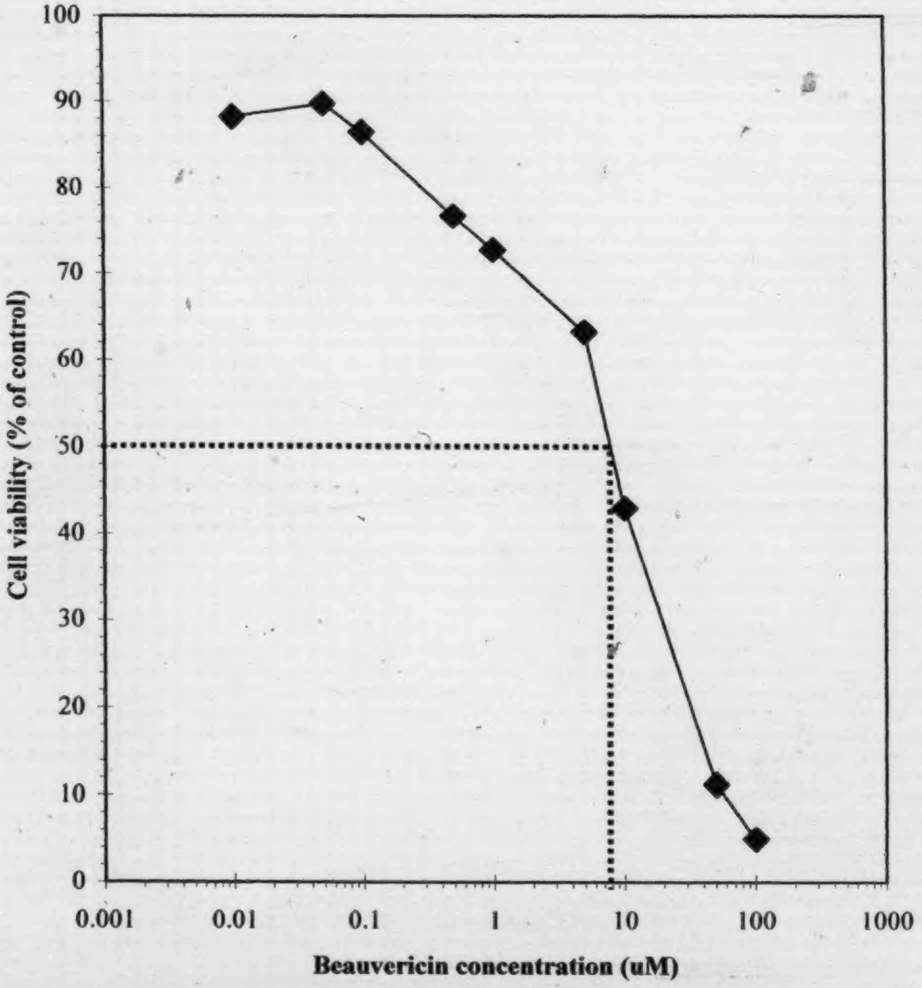

Figure 4.11. Log scale of pooled dose-response based on the mean cell viabilities obtained with standard beauvericin 48 hours post exposure to whitefly cell line Btb(Ba)97 Hunter-Polston and evaluated by 0.2% Trypan blue dye exclusion. [–♦–, 4 pooled trials (n = 12)]. CC_{50} interpolated (---) as 7.9 μM ± 2.0 μM.

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Chapter 5. Summary and conclusions

5.1. General discussion

5.1.1. Need for environmentally safer alternatives to chemical insecticides

The Green Revolution used selected cultivars of crop plants grown in conjunction with chemical fertilizers, pesticides and controlled irrigation to produce greater yields than traditional cultivars and methods (Rosset *et al.*, 2000). Initially these practices showed potential but became detrimental over time. Excessive application of chemical pesticides selected for resistance in insect populations, eliminated natural enemies leading to pest resurgence, caused secondary pest outbreaks and the evolution of non-pest to pest insect populations (Lacey and Goettel, 1995). In addition, use of chemical agents had other important consequences that affect humans and the environment such as persistent residues and contamination of groundwater (Lacey and Goettel, 1995; Inglis *et al.*, 2001). Due to limited alternatives, many methods of the Green Revolution are still employed in intensive cropping systems in agriculture and horticulture worldwide.

Western flower thrips, *Frankliniella occidentalis*, are a major pest in greenhouses (Lewis, 1997; Murphy *et al.*, 1998; Kumm, 2002). Characteristics of their biology that predispose them to become pests are their ability to cause direct feeding and oviposition damage, to spread virus diseases to crop plants; their tendency to spread and colonize rapidly, mainly by flight but also aboard planes and ships; and their ability to multiply rapidly in favourable environments (Lewis, 1997).

Though chemical insecticides are currently employed to manage thrips (Murphy *et al.*, 1998), safer alternatives must be developed to limit chemical use and to reduce the risk of environmental and human contamination (Liu *et al.*, 2002).

Entomopathogenic fungi, such as *Beauveria bassiana*, are more effective than other microbials as they do not need to be ingested and offer the only microbial-based control option against plant sucking insects (Lacey and Goettel, 1995). *Beauveria bassiana* exhibits a broad host range including western flower thrips (Fry *et al.*, 1999; Fry, 2003). It is naturally present in soil and is widely distributed geographically (St. Leger *et al.*, 1992). It is a cosmopolitan entomopathogen and although mitosporic; variants are known within and among isolates (St. Leger *et al.*, 1992; Maurer *et al.*, 1997; Castrillo and Brooks, 1998; Castrillo *et al.*, 1999). Genetic variability among isolates of *B. bassiana*, translates to varying degrees of virulence towards target insects. Inherent variability in the production of cuticle-degrading enzymes among isolates of *B. bassiana* has a strong influence on isolate virulence (Gupta *et al.*, 1992, 1994). Even though *B. bassiana* exhibits a broad host range, individual isolates have various degrees of host specificity (Goettel, 1992). As a result, individual isolates of *B. bassiana* are routinely screened against target insects for efficacy and virulence.

5.1.2. Research objectives

I examined *in vitro* properties of *B. bassiana* in relation to *F. occidentalis in vivo* virulence, and have developed *in vitro* tools to identify isolates of *B. bassiana* for high virulence to facilitate screening and to contribute to a greater understanding of the mechanism of *B. bassiana* pathogenesis for *F. occidentalis*. These tools can be considered from both macro- and micro-perspectives. For screening, analysis of colony growth morphology (macro-perspective) will help reduce processing time and labour intensity intrinsic to *in vivo* studies. Metabolite analysis (micro-perspective) increases screening efficiency, promotes a greater understanding of pathology and assists in microbial control registration for commercial use.

5.1.2.1. In vitro morphological characterization of Beauveria bassiana

This study is the first to characterize the virulence of *B. bassiana* isolates collected from both diseased and non-diseased insects and bioassayed against western flower thrips.

I demonstrate that *in vitro* growth analysis is useful in predicting degree of virulence against western flower thrips. Isolates of *B. bassiana* exhibiting good to excellent harvest quality, thin or few (i.e. powder-like conidia) mycelial fragments and none to moderate degree of submerged mycelia have a high probability of having high *in vivo* virulence. Isolates exhibiting poor to moderate harvest quality, thick mycelial fragments and a high degree of submerged mycelia will have a high probability of having low *in vivo* virulence.

5.1.2.2. Potentially novel role for oosporein in the life history of Beauveria bassiana

Results of *in vitro* growth analysis demonstrate that red pigmentation caused by oosporein in not associated with *in vivo* virulence and thus cannot by used as an indicator of such virulence. Oosporein, instead, may function as a photo-protectant while *B. bassiana* is in the vegetative state.

Based on the putative metabolic rate of an isolate or group of isolates and its/their ability to synthesize oosporein in the presence of light, I propose that they will produce oosporein at a specific time early in growth for a specific length of time to protect itself from potential damage caused by UV-irradiation.

My results further indicate that *B. bassiana* isolates capable of synthesizing oosporein and exposed to light, will express differing concentrations of oosporein depending on the isolate. Some isolates may repress oosporein production during the transition from vegetative to reproductive growth while others may continue to express oosporein once it has been induced. The fate of oosporein in cultures where colouration decreased in the medium over time is unknown though it may be degraded and/or re-absorbed by the isolate.

This is the first study to report that oosporein produced by *B. bassiana* may function in photo-protection. This idea is plausible based on Bandaranayake's (1998) view of UV-absorbing metabolites/compounds as nature's sunscreens (i.e. mycosporines). Oosporein may have some relation to mycosporines, which confer either a photo-protective role to vegetative mycelia or facilitate other aspects of fungal growth besides induction of sporulation as *B. bassiana* can sporulate in the dark.

5.1.2.3. Relationship between Beauveria bassiana virulence and beauvericin

Results of this study demonstrate that the five *B. bassiana* isolates examined, ranging from low to high virulence, all produced beauvericin. This suggests that beauvericin production and whole animal virulence may be uncoupled. My results also suggest a weak correlation between beauvericin production and whole animal virulence:

if beauvericin is present in high concentrations, the isolate will also probably exhibit a high degree of virulence. However, if beauvericin is present in low concentrations, it does not mean that the isolate will exhibit a low degree of virulence.

Uncoupling whole animal virulence from beauvericin production may provide flexibility in selecting isolates to control pests in different production systems. Because beauvericin is a common metabolite of many phytopathogenic and entomopathogenic fungi, toxicology studies must be carried out when considering whether to register *B. bassiana* for commercial use. Ideally, high virulence in concert with low beauvericin production precludes concerns about significant food contamination.

5.1.2.4. In vitro analysis of beauvericin using a whitefly cell line

The toxicity of beauvericin produced by various entomopathogenic and phytopathogenic fungi was evaluated on a whitefly *Bemisia argentifolii* (Btb(Ba)97 Hunter-Polston) cell line using Trypan blue dye exclusion *in situ*. This cell line, derived from embryonic tissue, was found to be sensitive to beauvericin in a dose- and time-dependent fashion. I demonstrated the CC_{50} after 48 h incubation to be comparable to that of other researchers using other cell lines.

5.1.2.5. Global evaluation of in vitro properties in relation to in vivo virulence

Analysis of colony growth morphology and beauvericin production can better predict *F. occidentalis in vivo* virulence. If an isolate exhibits the growth characteristics indicating high virulence and produces a high level of beauvericin, the isolate will probably be highly virulent *in vivo*. If an isolate exhibits the growth characteristics indicating low virulence and produces a low level of beauvericin, the isolate will probably have low virulence *in vivo*. An exception is when an isolate exhibits the growth characteristics indicating high virulence but produces a low level of beauvericin. Based on results of my studies, there is a good chance that these isolates will be highly virulent *in vivo* as emphasis is placed on analysis of colony growth morphology. With these isolates, other virulence factors such as production of cuticle degrading enzymes and other mycotoxins are likely to play a role in *F. occidentalis in vivo* virulence.

5.2. Conclusions

1. *In vitro* analysis of colony growth morphology for *B. bassiana* virulence is useful in predicting degree of *in vivo* virulence against western flower thrips.
2. Red pigmentation caused by oosporein production is not associated with *in vivo* virulence and thus cannot by used as an indicator of such virulence.
3. Oosporein may not have antibiotic activity but instead may function as a photo-protectant while *B. bassiana* is in the vegetative state.
4. Beauvericin production and *in vivo* virulence may be uncoupled. However there is a weak correlation between beauvericin production and *in vivo* virulence.
5. The whitefly embryonic cell line, Btb(Ba)97 Hunter-Polston, is sensitive to beauvericin in a dose- and time-dependent fashion and is comparable to results of other cell lines.

5.3. Future research

5.3.1. *In vitro morphological characterization of Beauveria bassiana*

1. **More isolates of *B. bassiana* should be evaluated for gross colony characteristics to further support the data presented.**

2. **Investigation of product formulation using selected isolates.**

In comparing isolates, I have identified those that perform better than or equivalent to the industry standard (GHA). However, it is important to consider the carrier agent and its effects on overall efficacy. Thus, it would be useful to incorporate these isolates with the carrier agent used for GHA and to compare them leveling *in vivo* studies.

5.3.2. *Potentially novel role for oosporein in the lifecycle of Beauveria bassiana*

1. **Replication of my experiments with additional isolates of *B. bassiana*.**

2. **Oosporein-infused culture filtrates should be sterilized via syringe filtration and subjected to spectrophotometric analyses to quantify oosporein production and to determine UV absorption maxima.**

3. **How does the possibility of photo-protection translate *in vivo*?**

4. **Confirm the role(s) oosporein has within the lifecycle of *B. bassiana* as a putative mycelial photo-protectant. Is oosporein related to other naturally occurring UV-absorbing compounds (mycosporines)?**

5.3.3. Relationship between Beauveria bassiana virulence and beauvericin

1. **Replication with the additional isolates.**

Due to time constraints, isolate GHA and SR-3 only were replicated. It would be useful to replicate the other isolates from trial 1.

2. **More isolates of *B. bassiana* should be evaluated for beauvericin production.**

3. **Comparison between *in vitro* and *in vivo* production.**

5.3.4. In vitro analysis of beauvericin using a whitefly cell line

1. ***In vivo* studies are needed to understand the effects of beauvericin in a more complex and dynamic environment.**

This is important because beauvericin will encounter cells organized into tissues or organs in whole animals where the number of interactions between cells is greater.

2. **Evaluation of beauvericin against a western flower thrips cell line.**

As a thysanopteran cell line is unavailable due to past unsuccessful attempts using late-staged embryos as a source, it would be useful to attempt using earlierstaged embryos between late anatrepsis and early intertrepsis at 18-21% total development time. Embryonic cells at this stage though partially differentiated, remain pleuripotent and are likely at an optimal stage for one to establish cell line(s). As time is required to establish a continuous cell line, beauvericin assayed on primary cells would also provide useful information.

5.4. A new future direction for this research

5.4.1. Aquaponics

Aquaponics is a relatively new greenhouse technology that combines aquaculture (fish-farming) and hydroponics (growing plants in a solution of water and nutrients without soil) within a re-circulating (closed) system. Elements a plant needs are added to water that is fed directly to plant roots sometimes within a medium such as perlite. Perlite provides plant support and keeps roots moist and aerated (Nelson, 2004). Hydroponics provides the plant with water, suitable nutrient ratios and optimal conditions for growth (Nelson, 2004). As fish digest their food and excrete waste products, a bioreactor containing a complex community of microorganisms converts these wastes so that the water becomes nutrient rich. Fish waste provides a food source for growing plants and plants provide a natural filter for water. This creates a mini-ecosystem where both plants and fish thrive (Nelson, 2004).

Chemical insecticides are not viable in this system as their incorporation lowers fish market value or may harm them. Using microbial control agents, such as *B. bassiana*, would be an ideal component of an integrated pest management program due to their selectivity and minimal environmental impact (Lacey and Goettel, 1995).

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